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## (54) Title: PROTEIN HAVING TPO ACTIVITY

The present invention relates to thrombopoietin (TPO) polypeptides having the biological activity of specifically stimulating or increasing platelet production comprising the amino acid sequence 1-332 of SEQ ID NO: 6 or a derivative thereof, DNA molecules encoding TPO polypeptides, processes for production of the polypeptides, antibodies specifically immunoreactive with the polypeptides, pharmaceutical compositions comprising the polypeptides, and methods for using the polypeptides in treatment of platelet disorders such

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### PROTEIN HAVING TPO ACTIVITY

This application is a continuation-in-part of U.S. Patent Application Serial No. (unknown) filed January 31, 1995, now pending, which is a continuation-in-part of U.S. Patent Application Serial No. 08/361,811 filed December 22, 1994, now pending, which is a continuation-in-part of U.S. Patent Application Serial No. 08/320,300 filed October 11, 1994, now pending, which is a continuation-in-part of U.S. Patent Application Serial No. 08/278,083 filed July 20, 1994, now pending, which is a continuation-in-part of U.S. Patent Application Serial No. 08/221,020 filed April 1, 1994, now abandoned, which is a continuation-in-part of U.S. Patent Application Serial No. 08/212,164 filed March 14, 1994 now abandoned.

#### FIELD OF THE INVENTION

This invention relates to a novel protein which has an activity to stimulate or increase platelet production *in vivo* in a specific manner or to enhance proliferation and differentiation of megakaryocyte progenitor cells, to a DNA sequence coding for said protein, and to a process for the production of the same.

# BACKGROUND OF THE INVENTION

Megakaryocytes are large cytoplasm-rich multinucleate cells which produce platelets and can be found Megakaryocytes originate from a mainly in bone marrow. pluripotent hematopoietic stem cell in bone marrow. primitive pluripotent stem cell differentiates to some degree into megakaryocyte progenitor cells, which are committed to the megakaryocytic lineage. Megakaryocyte progenitor cells differentiate into megakaryocytes. and proliferate Megakaryocytes further undergo polyploidization and cytoplasmic maturation and finally release their anuclear cytoplasmic fragments, namely platelets, into the circulation. Two to four thousands of platelets are on an average formed from one mature megakaryocyte. Although the platelet formation mechanism is still unclear in many points, it is considered that megakaryocytes are typically localized on the albuminal surface of the sinus endothelium in the bone marrow and produce cytoplasmic processes that extend into the sinusoid where they undergo fragmentation of platelets.

There are many ambiguous points regarding the mechanism for generating the platelets though it is likely that the megakaryocytes are locally present in the dermal layer of the skin of the venous sinus of the bone marrow and that the cytoplasm comes through the dermal skin to result in a cord-like projection on the internal wall of the venous sinus whereby the platelets are discharged.

It has been suggested that there is a specific function for the production and the control of the megakaryocyte hematopoiesis platelet production. In healthy human beings and animals, the number of the effective platelets is maintained though it has been known that, upon administration of an antiplatelet antibody to the healthy animals for example, the platelet number decrease promptly, then they begin to increase temporarily to larger than usual and, finally, they return to the normal level. Also in the clinical field, it has been known that a decrease in the number of the platelets thrombocytopenia and thrombocytosis even when the numbers of erythrocytes and leucocytes are at a normal level.

Incidentally, the most important function of a platelet is the production of a thrombus in a hemostatic mechanism. If the hemostatic mechanism does not function properly due to a decrease in the platelet number, a tendency towards hemostasis results.

The presence of a specific regulatory mechanism has been suggested with regard to megakaryocytopoiesis and thrombopoiesis. Platelets are maintained in effective numbers in healthy people and normal animals. However, it is known that, when an anti-platelet antibody is administered to a normal animal, only the number of platelets decreases sharply within a short period of time, starts to increase thereafter and temporarily exceeds the normal level, but finally returns to the normal level. It is known also in the clinical field that a decrease in the number of platelets (thrombocytopenia) or an increase in their number (thrombocytosis) occurs even under normal numbers of erythrocytes and leucocytes. however, success in the isolation and identification of a specific regulatory factor involved in platelet production (for example, like the case of erythropoietin in the erythrocyte formation) has not been reported.

Most important function of platelets is the formation of blood clot in the hemostatic mechanism. Bleeding tendency occurs when normal function of the hemostatic mechanism is spoiled by thrombocytopenia. In the field of radiotherapy and chemotherapy of cancers, thrombocytopenia caused by bone marrow suppression is a mortal complication, and platelet transfusion is applied to such patients in order to prevent bleeding tendency. Platelet transfusion is also applied to patients after bone marrow transplantation or of aplastic anemia.

Platelets for use in such platelet transfusion are prepared by plateletpheresis from blood of healthy blood donors, but such platelets for transfusion use have a short shelf life and a possibility of causing contamination by bacterial infection. The platelet transfusion also has a possible danger of exposing patients to dangerous viruses such as human immunodeficiency virus (HIV) or various hepatitis viruses, of inducing antibodies specific for a major

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histocompatibility antigen (HLA) of the transfused platelets or of causing graft versus host disease (GVHD) due to contaminated lymphocytes in the platelets for transfusion use.

In consequence, it will be of great benefit if intrinsic platelet formation can be stimulated in thrombocytopenic patients and, at the same time, their platelet transfusion dependency can be reduced. In addition, if thrombocytopenia in cancer patients undergoing radiotherapy or chemotherapy can be corrected or prevented, such treatments can be made more safer, the intensity of the treatment can possibly be increased, and further improvement of the anti-cancer effects can be expected.

For these reasons, a number of intensive studies have been made on the isolation and identification of specific regulatory factors involved in the regulation of megakaryocyte According to in vitro studies, and platelet production. regulatory factors governing megakaryocytopoiesis are roughly divided into the following two factors (cf. Williams et al., J. Cell. Physiol., vol.110, pp.101 - 104, 1982). Megakaryocyte colony stimulating factor (Meg-CSF) is a regulatory factor which stimulates proliferation and differentiation of CFU-MK to form megakaryocyte colonies in a semi-solid culture medium. The other regulatory factor, called megakaryocyte potentiating factor (Meg-Pot), megakaryocyte stimulating thrombopoiesis stimulating factor or the like, acts mainly upon immature or mature megakaryocytes thereby enhancing their differentiation and maturation. The Meg-Pot is detectable in combination with Meg-CSF activity in some cases. In addition, since platelet counts increased when serum collected from plasma experimentally thrombocytopenic animals was administered to other normal animals, it has been suggested that there is a humoral factor, called thrombopoietin (TPO), capable of promoting platelet production in vivo.

In recent years, some cytokines whose genes have been cloned were examined for their abilities to stimulate megakaryocytopoiesis and thrombopoiesis. Human IL-3 stimulated formation of human megakaryocyte colonies (Bruno et al., Exp. Hematol., vol.16, pp.371 - 377, 1988) and, at least in monkey, increased platelet counts (Donahue et al., Science, vol.241, p.1820, 1988). However, since IL-3 is a factor which exerts its effects upon proliferation and differentiation of all hematopoietic cells, it can be distinguished from specific regulatory factors controlling megakaryocytopoiesis and Human IL-6 did not show Meg-CSF platelet production. activity, but acted upon immature megakaryocytes and then enhanced their differentiation into mature megakaryocytes (Williams et al., Exp. Hematol., vol.18, p.69, 1990). In vivo administration of IL-6 induced platelet production and promoted both maturation and shifted to higher ploidy of bone marrow megakaryocytes in primates, but also caused side effects such as reduction of body weight, induction of acute phase protein (Asano et al., Blood, vol.75, pp.1602 - 1605, 1990; Stahl et al., Blood, vol.78, pp.1467 - 1475, 1991). Human IL-11 showed no Meg-CSF activity, but exerted Meg-Pot activity and promoted platelet production in mice (Neben et al., Blood, vol.81, pp.901 - 908, 1993). In addition, human LIF significantly increased platelet counts in primates (Mayer et al., Blood, vol.81, pp.3226 - 3233, 1993), but its in vitro action upon megakaryocytes was weak (Burstein et al., J. Cell. Physiol., vol.153, pp.305 - 312, 1992).

Although clinical application of these cytokines as platelet increasing factors is expected, their functions are not specific for the megakaryocyte lineage and they cause side effects. Therefore, development of a platelet increasing factor which is specific for the megakaryocyte-platelet system and causes less side effects has been called for in the clinical field.

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Meg-CSF, Meg-pot or TPO activity has been found in serum, plasma or urine of thrombocytopenic patients or animals, or in culture supernatant of certain human cultured cell lines. However, whether these activities are due to the presence of a single factor or a combination of several factors or whether they are different from known cytokines is presently unknown.

Hoffman et al. have found that sera of patients with aplastic anemia and amegakaryocytic thrombocytopenic purpura contained a Meg-CSF activity that significantly augmented human megakaryocyte colony formation (Hoffman et al., N. Eng. J. Med., vol.305, pp.533 - 538, 1981). Thereafter, Mazur et al. have reported that the Meg-CSF activity present in the serum of aplastic anemia patients was distinct from both IL-3 and GM-CSF (Mazur et al., Blood, vol.76, pp.290 - 297, 1990). Similar Meg-CSF activity has been found in sera of cancer patients receiving intensive cytotoxic chemotherapy and bone marrow-transplant patients (Mazur et al., Exp. Hematol., voi.12, pp.624 - 628, 1984; de Alarcon and Schmieder, Prog. Clin. Bio. Res., vol.215, pp.335 - 340, 1986). Hoffman et al. have reported that Meg-CSF with an apparent molecular weight of 46,000 was purified from plasma of patients with hypomegakaryocytic thrombocytopenia (Hoffman et al., J. Clin. Invest., vol.75, pp.1174 - 1182, 1985), but it was found by further studies that the material was not at a level of purity to allow accurate amino acid sequencing (Hoffman, Blood, vol.74, pp.1196 - 1212, 1989). A substance having TPOlike activity which enhanced incorporation of 75Seselenomethionine into newly forming platelets in mice has been partially purified from plasma of thrombocytopenic patients or urine of patients with idiopathic thrombocytopenic purpura (ITP), and apparent molecular weight of the plasmaderived factor was determined to be 40,000 (Grossi et al., Hematologica, vol.72, pp.291 - 295, 1987; Vannucchi et al., Leukemia, vol.2, pp.236 - 240, 1988).

Meg-CSF and TPO-like activities have also been detected in urine samples of patients with aplastic anemia and severe ITP (Kawakita et al., Br. J. Haematol., vol.48, pp.609 -615, 1981; Kawakita et al., Blood, vol.556 - 560, 1983). Kawakita et al. have further reported that the Meg-CSF activity found in the urine extract of aplastic anemia patients showed an apparent molecular weight of 45,000 by gel filtration under a dissociation condition (Kawakita et al., Br. J. Haematol., vol.62, pp.715 - 722, 1986). Erikson-Miller et al. have also reported on the purification of Meg-CSF from similar urinary samples, but with no information on its structure (Erikson-Miller et al., "Blood Cell Growth Factors: their present and future use in hematology and oncology" ed. by Murphy, AlphaMed Press, Dayton, Ohio, pp.204 - 220, 1992). Turner et al. have purified a megakaryocyte stimulating factor (MSF) having Meg-CSF activity from the urine of bone marrow transplant patients and cloned its gene (Turner et al., Blood, vol.78, p.1106 279a, 1991, (abstr., supple. 1)). This MSF has a molecular weight of 28,000 to 35,000. Identity of this factor with the Meg-CSF so far detected in serum and plasma samples of thrombocytopenic patients and its platelet increasing activity remain to be elucidated.

A substance having a TPO-like activity with a molecular weight of 32,000 has been purified from the culture supernatant of a human embryonal kidney-derived cell line (HEK cells) and its biological and biochemical properties have extensively been examined, but its structure is still unknown (McDonald et al., J. Lab. Clin. Med., vol.106, pp.162 - 174, 1985; McDonald, Int. J. Cell Cloning, vol.7, pp.139 - 155, 1989). On the contrary, other researchers have reported that the main activity in the conditioned medium of the HEK cells, which enhances megakaryocyte maturation in vitro, is due to known

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cytokines, namely IL-6 and EPO (Withy et al., J. Cell. Physiol., vol.15, pp.362 - 372, 1992).

With regard to animal-derived factors, Evatt et al. have reported that a TPO-like activity which enhanced incorporation of <sup>75</sup>Se-selenomethionine into newly forming platelets in rabbits and mice was found in plasma of thrombocytopenic rabbits induced by antiplatelet serum injection (Evatt et al., J. Lab. Clin. Med., vol.83, pp.364 - 371, 1974). In addition to this, a number of similar studies have been reported from the 1960's to 1970's (for example, Odell et al., Proc. Soc. Biol. Med., vol.108, pp.428 - 431, 1961; Evatt and Levin, J. Clin. Invest., vol.48, pp.1615 - 1626, 1969; Harker, Am. J. Physiol., vol.218, pp.1376 - 1380, 1970; Shreiner and Levin, J. Clin. Invest., vol.49, pp.1709 - 1713, 1970; and Penington, Br. Med. J., vol.1, pp.606 - 608, 1070). Evatt et al., and Hill and Levin have conducted partial purification of a TPO-like activity from plasma of thrombocytopenic rabbits (Evatt et al., Blood, vol.54, pp.377 - 388, 1979; Hill and Levin, Exp. Hematol., vol.14, pp.752 - 759, 1986). Thereafter, purification of this factor was continued, monitoring an activity to enhance differentiation and maturation of megakaryocytes in vitro, namely Meg-Pot activity, to reveal that this activity has an apparent molecular weight of 40,000 to 46,000 when determined by gel filtration (Keller et al., Exp. Hematol., vol.16, pp.262 - 267, 1988; Hill et al., Exp. Hematol., vol.20, pp.354 - 360, 1992). Since IL-6 activity was not detectable in plasma of rabbits with severe acute antiplatelet by thrombocytopenia induced administration, it was suggested that this TPO-like activity would be due to a factor other than IL-6 (Hill et al., Blood, vol.80, pp.346 - 351, 1992).

Tayrien and Rosenberg have also purified a factor having an apparent molecular weight of 15,000 from thrombocytopenic rabbit plasma and culture supernatant of

HEK cells, which stimulates production of platelet factor 4 in a rat megakaryocytic cell line, but with no information on its structure (Tayrien and Rosenberg, *J. Biol. Chem.*, vol.262, pp.3262 - 3268, 1987).

In addition, Nakeff has found a Meg-CSF activity in serum of thrombocytopenic mice induced by anti-platelet administration (Nakeff, "Experimental Hematology Today" ed. by Baum and Ledney, Springer-Verlag, NY, pp.111 - 123, 1977). On the other hand, sera from thrombocytopenic rabbits enhanced maturation of megakaryocytes (Keller et al., Exp. Hematol., vol.16, pp.262 - 267, 1988; Hill et al., Exp. Hematol., vol.17, pp.903 - 907, 1989) and stimulated morphological change of megakaryocytes into platelets (Leven and Yee, Blood, vol.69, pp.1046 - 1052, 1989), but had no detectable Meg-CSF activity.

Miura et al. have detected a Meg-CSF activity in plasma of rats rendered thrombocytopenic by sublethal whole body irradiation (Miura et al., Blood, vol.63, pp.1060 - 1066, 1984), and suggested that induction of the Meg-CSF activity in vivo is related to decreased megakaryocytes, but not decreased platelets, because this activity did not change by platelet transfusion (Miura et al., Exp. Hematol., vol.16, pp.139 - 144, 1988). Mazur and South have detected a Meg-CSF activity in serum of sublethally irradiated dogs and reported that this factor has an apparent molecular weight of 175,000 when measured by gel filtration (Mazur and South, Exp. Hematol., In addition to the above, vol.13, pp.1164 - 1172, 1985). serum-, plasma- and urine-derived factors have been reported by other investigators for example by Straneva et al. (Straneva et al., Exp. Hematol., vol.15, pp.657 - 663, 1987).

Thus, as described above, various activities that stimulate megakaryocytopoiesis and thrombopoiesis have been found in biological samples derived from thrombocytopenic patients and animals, but isolation of these factors, their

biochemical and biological identification and determination of their characteristics have not been achieved because of their extremely small contents in natural sources such as blood and urine.

# SUMMARY OF THE INVENTION

Objects of the present invention are to isolate a TPO protein from natural sources and identify it, the TPO protein having an activity to stimulate or increase platelet production in vivo and/or to enhance proliferation and differentiation of megakaryocyte progenitor cells (hereinafter, referred to as "TPO activity"); and to isolate a gene coding for the TPO protein and to provide a process for the production of said protein in a homogeneous quality and in a large quantity with recombinant DNA techniques. Success in accomplishing such objects will lead to the substitution or frequency-reduction of the currently used platelet transfusion, and such a novel protein will also be used for treatment and diagnosis of platelet disorders.

The present invention, therefore, relates to (i) a purified and isolated DNA sequence encoding a protein having TPO activity, which is selected from the group consisting of:

- (a) the DNA sequences shown in SEQ ID NOs: 194, 195 and 196, or complementary strands thereof; and
- (b) DNA sequences which hybridize under stringent conditions to the DNA sequences as defined in (a) or fragments thereof; and
- (c) DNA sequences which would hybridize to the DNA sequences as defined in (a) and (b), but for the degeneracy of the genetic code,
  - (ii) to a process for the production of a protein having TPO activity, which comprises the steps of:

growing, under suitable nutrient conditions, procaryotic or eucaryotic host cells transformed or transfected with said DNA sequence in a manner enabling expression of the protein; and

isolating the desired protein product obtained by expression of said DNA sequence, and

(iii) to a protein product obtained by expression in a procaryotic or eucaryotic host cell of said DNA sequence.

The present invention further relates to a pharmaceutical composition comprising an effective amount of said protein having TPO activity, and to a method for treating platelet disorders, especially thrombocytopenia, comprising administering said protein to patients having the disorders.

# BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows Sephacryl S-200HR gel filtration chromatography of Phenyl Sepharose 6 FF/LS F2 derived from XRP.

Figure 2 shows Capcell Pak C1 reverse phase chromatography of YMC-pack CN-AP TPO-active fraction derived from low molecular TPO sample (Sephacryl S-200HR F3) of XRP.

Figure 3 shows a SDS-PAGE analysis of Capcell Pak C1 TPO-active fraction (FA) from low molecular TPO sample of XRP.

Figure 4 shows peptide maps on C18 reverse phase HPLC of rat TPO isolated by SDS-PAGE. The peptide fragments were obtained by systematic hydrolysis with three proteases.

Figure 5 shows TPO activity derived from XRP in the rat CFU-MK assay system.

Figure 6 shows a construction of expression vector pEF18S.

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Figure 7 shows TPO activity in the culture supernatant of COS1 cells into which pEF18S-A2 $\alpha$  was introduced in the rat CFU-MK assay system.

Figure 8 shows TPO activity in the culture supernatant of COS1 cells into which pEF18S-HL34 was introduced in the rat CFU-MK assay system.

Figure 9 shows TPO activity in the culture supernatant of COS1 cells into which pHT1-231 was introduced in the rat CFU-MK assay system.

Figure 10a shows TPO activity in the culture supernatant of COS1 cells into which pHTF1 was introduced in the rat CFU-MK assay system.

Figure 10b shows TPO activity in the culture supernatant of COS1 cells into which pHTF1 was introduced in the M-07e assay system.

Figure 11 shows a restriction map of the  $\lambda$ HGT1 clone and construction of pHGT1 and pEFHGTE.

(E: EcoRI, H: HindIII, S: Sall)

Figure 12a shows TPO activity in the culture supernatant of COS1 cells into which pEFHGTE was introduced in the rat CFU-MK assay system.

Figure 12b shows TPO activity in the culture supernatant of COS1 cells into which pEFHGTE was introduced in the M-07e assay system.

Figure 13a shows TPO activity in the culture supernatant of COS1 cells into which pHT1-211#1, pHT1-191#1, or pHT1-171#2 was introduced in the rat CFU-MK assay system.

Figure 13b shows TPO activity in the culture supernatant of COS1 cells into which pHT1-163#2 was introduced in the rat CFU-MK assay system.

Figure 14 shows TPO activity in the culture supernatant of COS1 cells into which pHT1-211#1, pHT1-

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191#1, pHT1-171#2, or pHT1-163#2 was introduced in the M-07e assay system.

Figure 15 is a chromatogram of a reverse phase chromatography (Vydac C4 column) for purification of human TPO from the culture supernatant of CHO cells into which pDEF202-hTPO-P1 has been introduced and allowed the TPO to be expressed.

Figure 16 is a photograph showing the SDS-PAGE separation of the human TPO purified from the culture supernatant of CHO cells into which pDEF202-hTPO-P1 has been introduced and allowed the TPO to be expressed.

Figure 17 is a chromatogram of a reverse phase chromatography for purification of human TPO from <u>F. coli</u> into which pCFM536/h6T(1-163) has been introduced and allowed the TPO to be expressed.

Figure 18 is a photograph showing the SDS-PAGE separation of the variant human TPO, h6T(1-163) isolated and purified from <u>E. coli</u> into which pCFM536/h6T(1-163) has been introduced and allowed the TPO to be expressed.

Figure 19 shows an elution pattern of hTPO163 on Superdex 75 pg column with regard to the purification of the hTPO 163 from the culture supernatant, as a starting material, prepared by transfection of the human TPO expression plasmid pDEF202-hTPO163 into CHO cells. The protein amount was determined at 220 nm.

Figure 20 shows an SDS-PAGE analysis of the standard hTPO163 eluted from Superdex 75 pg column upon the purification of the hTPO163 from the culture supernatant, as a stating material, prepared by transfection of the human TPO expression plasmid pDEF202-hTPO163 into CHO cells. The hTPO163 was silver-stained on the gel.

Figure 21 shows a structure of the expression vector pSMT201.

Figure 22 shows a graph showing the TPO activity as determined by M-07e assay, in the culture supernatant of the COS7 that  $\beta$ GL-TPO, N3/TPO or 09/TPO has been introduced into and then expressed.

Figure 23 is a graph showing a TPO activity, as determined by M-07e assay, in the supernatants of COS7 cell cultures that an insertion or deletion derivative of human TPO has been introduced into and expressed.

Figure 24 shows increased platelet count in mice administered TPO via intravenous and subcutaneus injection.

Figure 25 shows dose dependent increased platelet count in mice following subcutaneous injection of TPO.

Figure 26 shows TPO induced increase in platelet count following treatment of mice with 5-FU to induce thrombocytopenia.

Figure 27 shows TPO induced increase in platelet count following treatment of mice with nimustine hydrochloride to induce thrombocytopenia.

Figure 28 shows TPO induced increase in platelet count thrombocytopenic mice following bone marrow transplant.

Figure 29 shows TPO induced platelet count following x-ray irradiation of mice to induce thrombocytopenia.

Figure 30 shows dose-dependent increase in platelet count following administration of truncated TPO (amino acids 1-163 in SEQ ID NO: 6).

Figure 31 shows increase in platelet count following administration of truncated TPO (amino acids 1-163 in SEQ ID NO: 6) following administration of nimustine hydrochloride to induce thrombocytopenia.

Figure 32 shows that increasing concentrations of MpI-X added to the human megakaryocyte culture system increasingly block megakaryocyte development.

Figure 33 describes TPO activity, as determined by M-07e assay, of TPO derivatives [Met-2, Lys-1, Ala1, Val3, Alg133]TPO(1-163), [Met-2, Lys-1, Ala1, Val3, Pro148]TPO(1-163) and [Met-2, Lys-1, Ala1, Val3, Arg115]TPO(1-163) expressed in *E. coli*.

Figure 34 describes TPO activity, as determined by M-07e assay, of TPO derivatives [Met-2, Lys-1, Ala1, Val3, Alg129]TPO(1-163), [Met-2, Lys-1, Ala1, Val3, Arg143]TPO(1-163), [Met-2, Lys-1, Ala1, Val3, Leu82]TPO(1-163), [Met-2, Lys-1, Ala1, Val3, Leu146]TPO(1-163) and [Met-2, Lys-1, Ala1, Val3, Arg59]TPO(1-163) expressed in  $E.\ coli.$ 

# DETAILED DESCRIPTION OF THE INVENTION

Specifically provided according to the present invention are thrombopoietin (TPO) polypeptides having the biological activity of specifically stimulating or increasing platelet production and comprising the amino acid sequence 1 to 332 of SEQ ID NO: 6 or derivatives thereof. polypeptides include those which consist of the amino acid sequences 1 to 163 of SEQ ID NO: 6; 1 to 232 of SEQ ID NO: 6; 1 to 151 of SEQ ID NO: 6; the mature sequence of amino acids of SEQ ID NOs: 2, 4 and 6; including those having from 1 to 6 NH2 Additional illustrative terminal amino acids deleted. polypeptides of the invention include [Thr<sup>33</sup>, Thr<sup>333</sup>, Ser<sup>334</sup>, lle335, Gly336, Tyr337, Pro338, Tyr339, Asp340, Val341, Pro342, Asp343, Tyr344, Ala345, Gly346, Val347, His348 His349, His350, His351, His352, His353]TPO, [Asn25, Lys231, Thr333 Ser334 lle335 Gly336 Tyr337 Pro338 Tyr339 Asp340, Val341, Pro342, Asp343, Tyr344, Ala345, Gly346, Val347, His348, His349, His350, His351, His352, His353]TPO, [Asn<sup>25</sup>]TPO and [Thr<sup>33</sup>]TPO. Other polypeptides of the invention include derivatives of a TPO polypeptide  $[\Delta His^{33}]TPO(1-163), [\Delta Arg^{117}]TPO(1-163), [\Delta Gly^{116}]TPO(1-163), [\Delta Gly^{116}]TPO(1-1$ 163), [His<sup>33</sup>, Thr<sup>33</sup>', Pro<sup>34</sup>]TPO(1-163), [His<sup>33</sup>, Ala<sup>33</sup>',

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Pro<sup>34</sup>]TPO(1-163), [His<sup>33</sup>, Gly<sup>33</sup>', Pro<sup>34</sup>, Ser<sup>38</sup>]TPO(1-163), [Gly<sup>116</sup>, Asn<sup>116</sup>', Arg<sup>117</sup>]TPO(1-163), [Gly<sup>116</sup>, Ala<sup>116</sup>', Arg<sup>117</sup>]TPO(1-163), [Gly<sup>116</sup>, Gly<sup>116</sup>', Arg<sup>117</sup>]TPO(1-163), [Ala<sup>1</sup>, Val<sup>3</sup>, Arg<sup>129</sup>]TPO(1-163), [Ala<sup>1</sup>, Val<sup>3</sup>, Arg<sup>133</sup>]TPO(1-163), [Ala<sup>1</sup>, Val<sup>3</sup>, Arg<sup>143</sup>]TPO(1-163), [Ala<sup>1</sup>, Val<sup>3</sup>, Leu<sup>82</sup>]TPO(1-163), [Ala<sup>1</sup>, Val<sup>3</sup>, Leu<sup>146</sup>]TPO(1-163), [Ala<sup>1</sup>, Val<sup>3</sup>, Pro<sup>148</sup>]TPO(1-163), [Ala<sup>1</sup>, Val<sup>3</sup>, Arg<sup>59</sup>]TPO(1-163), and [Ala<sup>1</sup>, Val<sup>3</sup>, Arg<sup>115</sup>]TPO(1-163).

The TPO polypeptides of the invention also include those which are covalently bonded to a polymer, preferably polyethylene glycol. The TPO polypeptides of the invention may further comprise the amino acids [Met<sup>-2</sup>-Lys<sup>-1</sup>], [Met<sup>-1</sup>] or [Gly<sup>-1</sup>]. DNAs provided by the invention include those which encode the TPO polypeptide and derivatives described above and are suitably provided as cDNA, genomic DNA and manufactured DNAs.

Also provided by the invention are processes for producing a TPO polypeptide as described above comprising the steps of expressing a polypeptide encoded by a DNA of the invention in a suitable host, and isolating said TPO polypeptide. Where the TPO polypeptide expressed is a Met<sup>-2</sup>-Lys<sup>-1</sup> polypeptide, such processes can further include the step of cleaving Met<sup>-2</sup>-Lys<sup>-1</sup> from said isolated TPO polypeptide.

Also provided by the invention are processes for producing TPO polypeptides such as glutathione-S-transferase (GST) fusion polypeptides. DNA encoding an amino terminal GST polypeptide, a thrombin recognition peptide and a TPO polypeptide is introduced into a suitable host, the fusion polypeptide is isolated and the GST moiety removed by treatment with thrombin. Resulting TPO polypeptides are of the [Gly-1] structure.

Additionally provided are procaryotic or eucaryotic host cells transformed or transfected with a DNA sequence according to the invention in a manner enabling said host cell

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to express a polypeptide having the biological activity of specifically stimulating or increasing platelet production.

Pharmaceutical compositions of the invention comprise an effective amount of a TPO polypeptide or derivative in combination with a pharmaceutically acceptable carrier and are susceptible to use in treatment of platelet disorders, particularly the treatment of thrombocytopenia, such as is induced by chemotherapy, radiotherapy or bone marrow transplantation. Corresponding treatment methods are provided by the invention.

Finally, the present invention provides antibodies specifically immunoreactive with TPO polypeptides and derivatives thereof as described above. Such antibodies are useful in methods for isolation and quantification of TPO polypeptides of the invention.

According to the present invention, there is provided a novel DNA sequence which encodes a protein having a TPO activity (referred to as "DNA sequence of the present invention", hereinafter). The DNA sequence of the present invention includes a DNA sequence which encodes the amino acid sequence shown in SEQ ID NO: 2, 4 or 6 of the Sequence Listing attached hereto.

Also included in the DNA sequence of the present invention is a DNA sequence which encodes a partly modified (substitution, deletion, insertion or addition) version of the aforementioned amino acid sequence shown in SEQ ID NO: 2, 4 or 6, provided that such modifications do not spoil the TPO activity. That is, DNA sequences coding for TPO derivatives are also included in the present invention.

In other words, the DNA sequence of the present invention includes DNA sequences which encode protein molecules whose amino acid sequences are substantially the amino acid sequences shown in SEQ ID NO: 2, 4 or 6. The wording "amino acid sequences are substantially the amino

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acid sequences shown in SEQ ID NO: 2, 4 or 6" as used herein means that said amino acid sequences include those represented by SEQ ID NO: 2, 4 or 6 as well as those represented SEQ ID NO: 2, 4 or 6 which have partial modification therein such as substitution, deletion, insertion, addition or the like, provided that such modifications do not spoil the TPO activity.

Further, the DNA sequence of the present invention consists essentially of a DNA sequence encoding a protein having a TPO activity.

The wording "a DNA sequence which encodes the amino acid sequence" includes all the DNA sequences which may have degeneracy in nucleotide sequences.

The DNA sequence of the present invention also comprises the following sequences:

- (a) DNA sequences represented by SEQ ID No: 7, 194, 195 and 196, or complementary strands thereof,
- (b) DNA sequences which hybridize under stringent conditions to the DNA sequences as defined in (a) or fragments thereof, or
- (c) DNA sequences which would hybridize to the DNA sequences as defined in (a) and (b), but for the degeneracy of the genetic code.

In other words, the DNA sequence of the present invention also comprises the following sequences:

(a) DNA sequence which is integrated in the Vector pEF18S-A2α (deposit No. FERM BP-4565) carried by an *E. coli* strain DH5, a Vector pHT1-231 (deposit No. FERM BP-4564) carried by an *E. coli* strain DH5, a Vector pHTF1 (deposit No. FERM BP-4617) carried by an *E. coli* strain DH5, a Vector pHGT1 (deposit No. FERM BP-4616) carried by an *E. coli* strain DH5, and encodes the amino acid sequence of a protein having the TPO activity; or

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(b) DNA sequences which hybridize (under stringent conditions) to the DNA sequences defined in (a) or fragments thereof, and encode the amino acid sequence of a protein having the TPO activity.

As used herein, representative "stringent" hybridization conditions are those employed in the examples addressing PCR amplification of DNAs of the invention using degenerate and/or unique sequence oligonucleotide primers (probes). See also for example, Chapters 11 and 14 of Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) and Unit 2.10 in Current Protocols in Molecular Biology, Ausubel et al., Eds., Current Protocols, USA, 1993).

Also included in the DNA sequence of the present invention is a DNA sequence which encodes a protein having TPO activity and comprises the nucleotide sequence encoding positions 1-163 of the amino acid sequence represented by SEQ ID NO: 6.

Such DNA sequences may be supplemented with a restriction enzyme cleavage site and/or an additional DNA sequence at the initiation, termination or intermediate site which facilitate construction of readily expressed vectors. When a non-mammalian host is used, a preferred codon for the gene expression in the host may be incorporated.

An example of the DNA sequence of the present invention is a cDNA molecule which is obtained by preparing mRNA from cells of mammals, including human, and then screening the cDNA in the usual way from a cDNA library prepared by a known method. Sources of the mRNA of this case include cells of a rat hepatocyte-derived cell line McA-RH8994, HTC cells, H4-II-E cells, rat liver, kidney, brain and small intestine human liver and the like.

Another example of the DNA sequence of the present invention is a genomic DNA molecule which is obtained

by screening it in the usual way from a genomic library prepared by a known method from cells of mammals including human. Sources of the genomic DNA of this case include chromosomal DNA preparations obtained from human, rat, mouse and the like.

A DNA sequence which encodes a TPO derivative may be obtained from the thus obtained cDNA sequence that encodes a protein having TPO activity, by modifying the cDNA sequence making use of the known site-directed mutagenesis thereby effecting partial modification of the corresponding amino acid sequence.

Having elucidated the amino acid sequence or DNA sequence of a protein having TPO activity in the present invention, a DNA sequence which encodes a partially modified amino acid sequence can be obtained easily by chemical synthesis.

The DNA sequence of the present invention is a useful material for the large scale production of a protein having TPO activity making use of various recombinant DNA techniques.

Also, the DNA sequence of the present invention is useful as a labeled probe for the isolation of a gene which encodes a TPO-related protein, as well as cDNA and genomic DNA coding for TPO of other mammalian species. It is also useful in the gene therapy of human and other mammalian species. In addition, the DNA sequence of the present invention is useful for the development of a transgenic mammalian species which can be used as a eucaryotic host for large scale production of TPO (Palmiter et al., Science, vol.222, pp.809 -814, 1983).

Also provided by the present invention are a vector in which the aforementioned DNA sequence coding for a protein having TPO activity is integrated, host cells transformed with said vector and a process for the production of a protein having

TPO activity which comprises culturing said host cells and separating and purifying the expressed protein having TPO activity.

Examples of host cells useful in this case include cells of prokaryotes, such as, *E. coli* and the like, and of eukaryotes, such as yeasts, insects, mammals and the like. Illustrative examples of mammalian cells include COS cells, Chinese hamster ovary (CHO) cells, C-127 cells, baby hamster kidney (BHK) cells and the like. Illustrative examples of yeasts include, a baker's yeast (*Saccharomyces cerevisiae*), a methanol assimilating yeast (*Pichia pastoris*) and the like. Illustrative examples of insect cells include, silkworm cultured cells and the like.

regard to vectors for use in With transformation of these host cells, pKC30 (Shimatake H. and M. Rosenberg, Nature, 292, 128 - 132, 1981), pTrc99A (Amann E. et al., Gene, 69, 301 - 315, 1988) or the like may be used for the transformation of E. coli cells. For transforming mammalian cells, pSV2-neo (Southern and Berg, J. Mol. Appl. Genet., 1, 327 - 341, 1982), pCAGGS (Niwa et al., Gene, 108, 193 - 200, 1991), pcDL-SRa296 (Takebe et al., Mol. Cell. Biol., 8, 466 - 472, 1988) or the like may be used. As for yeast cells, pG-1 (Schena M. and Yamamoto K.R., Science, 241, 965 -967, 1988) or the like may be used. A transfer vector for recombinant virus construction, for example, pAc373 (Luckow et al., Bio/Technology, 6, 47 - 55, 1988) may be used for the transformation of silkworm cells.

As occasion demands, each of these vectors may contain a replication origin, selection marker(s), a promoter and the like, as well as an RNA splice site, a polyadenylation signal and the like in the case of vectors for use in eukaryotic cells.

With regard to the replication origin, a sequence derived from, for example, SV40, adenovirus, bovine papilloma

virus or the like may be used in vectors for mammalian cells. A sequence derived from ColE1, R factor, F factor or the like may be used in vectors for  $E.\ coli$  cells. A sequence derived from 2  $\mu m$  DNA, ARS1 or the like may be used in vectors for yeast cells.

As to promoters for gene expression, those which are derived from, for example, retrovirus, polyoma virus, adenovirus, SV40 and the like may be used in vectors for mammalian cells. A promoter derived from bacteriophage  $\lambda$ , for example, trp, lpp, lac or tac promoter, may be used in vectors for E. coli cells. ADH, PHO5, GPD, PGK or MAF $\alpha$  promoter may be used in vectors for baker's yeast cells, and AOX1 promoter or the like in vectors for methanol assimilating yeast cells. A promoter derived from a nuclear polyhedrosis virus may be used in vectors for silkworm cells.

Typical examples of selection markers useful in vectors for mammalian cells include a neomycin (neo) resistance gene, a thymidine kinase (TK) gene, a dihydrofolate reductase (DHFR) gene, an E. coli xanthine-guanine phosphoribosyltransferase (Ecogpt) gene and the like. Illustrative examples of selection markers useful in vectors for E. coli cells include a kanamycin resistance gene, an ampicillin resistance gene, a tetracycline resistance gene and the like, and those for yeast cells include Leu2, Trp1, Ura3 and the like genes.

Production of a protein having TPO activity making use of appropriate combinations of these host-vector systems may be performed by transforming appropriate host cells with a recombinant DNA obtained by inserting the gene of the present invention in an appropriate site of the aforementioned vector, culturing the resulting transformant and then separating and purifying the polypeptide of interest from the resulting cells, or culture medium or filtrate. Commonly used

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means and procedures may be applied to this process in combination.

When expressing the gene of interest, the original signal sequence may be modified or replaced by a signal sequence derived from another protein in order to obtain homogeneous N-terminal of the expressed product. Homogenization of the N-terminal may be carried out by modification (substitution or addition) of the amino acid residues in the N-terminal or its vicinity. In the case of expression by using *E. coli*, as a host cell, for example, lysine residue may be further supplemented in addition to methionine residue.

The novel protein of the present invention having TPO activity (to be referred to as "protein of the present invention" hereinafter) includes proteins each of which comprising the amino acid sequence shown in SEQ ID NO: 2, 4 or 6. TPO derivatives whose amino acid sequences are partially modified (substitution, deletion, insertion or addition) are also included in the present invention, provided that the TPO activity is not spoiled by the modification.

In other words, the protein of the present invention includes protein molecules whose amino acid sequences are substantially the amino acid sequences shown in SEQ ID NO: 2, 4 or 6.

The wording "amino acid sequences are substantially the amino acid sequences shown in (or represented by) SEQ ID NO: 2, 4 or 6" as used herein means that said amino acid sequences include those shown in SEQ ID NO: 2, 4 or 6 as well as those shown in SEQ ID NO: 2, 4 or 6 which have partial modification therein such as substitution, deletion, insertion, addition and the like, provided that such modifications do not spoil the TPO activity.

The protein of this invention includes a protein containing the positions 7-151 of the amino acid sequence

shown in SEQ ID NO: 6 and having a TPO activity. Also included in the protein of the present invention is a protein which has TPO activity and comprises positions 1-163 of the amino acid sequence represented by SEQ ID NO: 6.

Examples of other TPO derivatives of the present invention include a derivative whose stability and durability in vivo were improved by amino acid modification (substitution, deletion, insertion or addition), a derivative in which at least one potential glycosylation was changed by deletion or addition, a derivative in which at least one cysteine residue was deleted or substituted by other amino acid residue (alanine or serine residue for example).

Preferably, the protein of the present invention is characterized in that it is separated and purified from host cells transformed with a recombinant vector containing a cDNA molecule, a genomic DNA molecule or a DNA fragment obtained by chemical synthesis.

When intracellular expression is effected using a bacterium such as *E. coli* as the host, a protein in which an initiation methionine residue is added to the N-terminal side of a protein molecule having TPO activity is obtained, which is also included in the present invention. Depending on the host used, the produced protein having TPO activity may or may not be glycosylated, and each of such cases is included in the protein of the present invention.

The protein of the present invention also includes naturally occurring TPO-active proteins purified and isolated from natural sources such as cell culture medium having TPO activity or human urine, serum and plasma.

A process for the purification of TPO from such natural sources is also included in the present invention. Such a purification process may performed by employing one of or a combination of usually used protein purification steps such as an ion exchange chromatography, a lectin affinity

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chromatography, a triazine dye affinity chromatography, a hydrophobic interaction chromatography, a gel filtration chromatography, a reverse phase chromatography, a heparin affinity chromatography, a sulfated gel chromatography, a hydroxylapatite chromatography, isoelectric focussing chromatography, a metal chelating chromatography, a preparative electrophoresis, an isoelectric focussing gel electrophoresis and the like. A purification process in which certain techniques are used in combination making use of physicochemical properties of TPO which can be deduced from the Examples of this specification is also included in the In addition, an antibody affinity present invention. chromatography to which an antibody capable of recognizing TPO is applied may also be used. Furthermore, it has been found that TPO is the ligand for Mpl (de Sauvage et al., Nature 369: 533-538 (1994); Bartley et al., Cell 77: 1117-1124 (1994); Kaushansky et al., Nature 369: 565-568 (1994)), by which the TPO may be purified using an affinity gel column that the MpI has been coupled to a resin. More particularly, an example of the column is an Mpl-X column which is prepared by coupling a resin with the extracellular region of Mpl (Mpl-X) produced by recombinant DNA techniques using CHO cell as a host (Bartley et al. (1994), supra).

As disclosed herein, the TPO polypeptides of this invention are further characterized by the ability to bind to the Mpl receptor and specifically to the extracellular (soluble) domain thereof.

Also included in the present invention is a protein encoded by a DNA moiety which is complementary to the protein-coding strand of a human cDNA or genomic DNA sequence of TPO gene, namely a "complementary inverted protein" disclosed by Tramontano et al. (Nucl. Acids Res., vol.12, pp.5049 - 5059, 1984).

Also included in the present invention is a protein of the present invention which is labeled with a detectable marker, for example, 1251 labeling or biotinylation, thus rendering possible provision of a reagent which is useful for the detection and quantification of TPO or TPO receptor-expressing cells in solid samples such as tissue and liquid samples such as blood, urine and the like.

The biotinylated protein of the present invention is useful in the case of its binding to immobilized streptavidin in order to remove megakaryoblasts from bone marrow at the time of autogenous bone marrow transplantation. It is also useful in the case of its binding to immobilized streptavidin in order to concentrate autogenous or allogenic megakaryocytic cells at the time of autogenous or allogenic bone marrow transplantation. A conjugate of TPO with a toxin such as ricin, diphtheria toxin or the like or with a radioactive isotope is useful in antitumor therapy and in conditioning for bone marrow transplantation.

The present invention also provides a nucleic acid material which is useful when labeled with a detectable marker including a radioactive marker or a non-radioactive marker such as biotin or when it is used in a hybridization procedure for the detection of the position of human TPO gene and/or its related gene family on a chromosomal map. Such a material is also useful for the confirmation of human TPO gene disorders at the DNA level and can be used as a genetic marker for the confirmation of adjoining genes and their disorders.

Also included in the present invention is a pharmaceutical composition which contains a therapeutically effective amount of the protein of the present invention together with a useful and effective diluent, antiseptic agent, solubilizing agent, emulsifying agent, adjuvant and/or carrier. The term "therapeutically effective amount" as used herein means an amount which provides a therapeutic effect for

specified conditions and routes of administering conditioning. Such a composition is used in the form of liquid, freeze-dried or dried preparation and comprises a diluent selected from various buffers (Tris-HCl, acetate and phosphate for example) having various pH values and ionic strengths, a surface adsorption preventing additive such as albumin or gelatin, a surface active agent such as Tween 20, Tween 80, Pluronic F68 or bile acid salt, a solubilizing agent such as glycerol or polyethylene glycol, an antioxidant such as ascorbic acid or sodium metabisulfite, an antiseptic agent such as thimerosal, benzyl alcohol or paraben and a vehicle or tonicity agent such as lactose or mannitol. Also used is a composition comprising covalent bonding of the protein of the present invention to a polymer such as polyethylene glycol, chelation of the protein with metal ions, incorporation of the protein into a granular preparation, or on the surface, consisting of a polymer compound such as polylatic acid, polyglycolic acid or hydrogel, liposomes, into incorporations of the protein microemulsion, micelles, single or multilayer vesicles, red cell ghosts or spheroplasts. Such a composition will exert influences upon physical conditions, solubility, stability, in vivo releasing rate and in vivo clearance of TPO. Selection of the composition may be decided depending on the physical and chemical properties of the used TPO-active protein. included in the present invention is a granular composition in which granules are coated with a polymer such as poloxamer or poloxamine and TPO which binds to antibodies for tissue specific receptors, ligands or antigens or to tissue specific receptor ligands. Other examples of the composition of the present invention are those which are in the form of granules, have a protective coating and contain a protease inhibitor or a penetration enhancer, for use in various routes of administration such as parenteral, pulmonary, transnasal and oral administration.

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The pharmaceutical composition comprising the protein of the present invention may be administrated several times per day, usually in an amount of  $0.05\mu g$  to 1mg/kg (as TPO protein) of body weight depending on conditions and sex of a patient, administration route and the like.

The pharmaceutical composition containing the protein of the present invention can be administered at a dose of 25,000-500,000,000 of the active ingredient (in terms of the relative activity by the M-07e assay which will be given later) per kg of body weight for one to several times a day and for one to seven days a week depending upon the symptoms, sex and route of administration.

The present inventors have confirmed that, with respect to the C-terminal site of the human TPO, the activity is maintained even when the amino acid residues up to the 152nd amino acid sequence shown in SEQ ID NO: 6 are deleted and that, with respect to the N-terminal site, the activity is maintained even when the amino acid residues up to the 6th amino acid sequence are deleted.

Accordingly, protein having TPO activity, containing the 7th to 151th amino acid sequence of SEQ ID NO: 6 and being modified (substitution, deletion, insertion or addition) in other parts may be preferably used as the effective ingredient of the present invention as well. More preferred TPO derivative is that having the 1st to 163rd amino acid sequence of SEQ ID NO: 6.

A composition which contains at least one of additional hematopoietic factors such as EPO, G-CSF, GM-CSF, M-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, LIF and SCF, in addition to the protein of the present invention, is also included in the present invention.

The protein of the present invention is useful for the treatment of various thrombocytopenic diseases, either

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alone or in combination with other additional hematopoietic factors. Examples of other additional hematopoietic factors include EPO, G-CSF, GM-CSF, M-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, LIF and SCF.

There are many diseases characterized by platelet disorders such as thrombocytopenia due to damages in the production of platelets or reduction in life of platelets (caused by destruction or consumption of platelets), which can be treated with the protein of the present invention. For example, it can be used for the enhancement of the restoration of platelets in a thrombocytopenic patient due to congenital Fanconi anemia or aplastic anemia caused by chemotherapy or irradiation, myelodysplastic syndrome, acute myelocytic leukemia, aplastic crisis after bone marrow transplantation. It is also useful in the treatment of thrombocytopenia due to diminished production of TPO. Thrombocytopenia due to reduction in life of platelets or megakaryocytes includes idiopathic thrombocytopenic purpura, hypoplastic anemia, acquired immunodeficiency syndrome (AIDS), disseminated intravascular coagulation syndrome and thrombotic thrombocytopenia. In addition, it is also useful in the case of autotransfusion of platelets, in which TPO is administered to a patient prior to a surgical operation in order to increase the number of the patient's own platelets and the thus increased platelets are used as transfusion platelets at the time of the operation.

Other use of the protein of the present invention is treatment of diseases resulting from temporal deletion or damage of platelets caused by other chemical or pharmaceutical drugs or curative treatments. TPO can be used for the purpose of enhancing release of new "intact" platelets in such patients.

The present invention further relates to an antibody specific for TPO. The protein of the present invention can be used as antigens, and the corresponding antibodies include both monoclonal and polyclonal antibodies and chimeric antibodies, namely "recombinant" antibodies, prepared by To prepare such an anti-TPO antibody, conventional methods. human TPO itself can be used as the antigen or, alternatively, a partial peptide of human TPO can be utilized as the antigen. Where an antibody to the antigen of such a peptide is utilized, the epitope can be defined by specifying the antigen region. On the other hand, where an antibody to the antigen protein (TPO) itself is utilized, the antigen region can be clarified by analyzing the epitope of the antibody. In such cases, these antibodies each having the thus-clarified epitope can be utilized to fractionate, detect, quantitate and purify various TPOs which differ in their properties such as the kind of the sugar chains added, the length of the peptide chains, etc.

A TPO peptide containing the thus-selected amino acid sequence is synthesized, and bonded to a suitable carrier KLH (key albumin, example. for protein. limpethemocyanine) or the like, by covalent bonding to prepare Alternatively, a peptide of a an immunoreactive antigen. multiple antigen peptide (MAP) type is produced to be an antigen, according to the Tam's method (Proc.Natl.Acad.Sci. Then, with the thus-prepared (USA) 85:5409-5413, 1988). antigen along with an adjuvant or the like, immunized are mammals, birds, etc. which are generally utilized to make them produce antibodies, such as rabbits, mice, rats, goats, sheep, chickens, hamsters, horses, guinea pigs, etc. From the thus-immunized animal, recovered was its anti-serum and cells, from which a polyclonal antibody is obtained. Where a peptide is used as the antigen, the epitope can be defined by specifying the antigen region. In this case, the regions of various TPO molecules having different molecular weights are

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screened and identified by immunological engineering. As one example, a peptide-deleted region can be screened and identified. In addition, an antibody gene or a part of the gene is cloned from the cells expressing the intended antibody to obtain an antibody molecule that has been expressed by genetic engineering.

In comparison with a polyclonal antibody which generally contains various antibodies specific for various antigenic determinants (epitopes), a monoclonal antibody is specific for a single antigenic determinant on the antigen. A TPO-specific antibody is useful in improving selectivity and specificity of an antigen-antibody reaction-aided diagnosis and an analytical assay method and in performing separation and purification of TPO. In addition, such antibodies can be applied to the neutralization or removal of TPO from serum. Monoclonal antibodies are also useful for detection and quantification of TPO in, for example, serum of whole blood.

The anti-human TPO antibody of the present invention can be used as the ligand in affinity chromatography for purification and isolation of human TPO. antibody so as to make it useful in such affinity chromatography, any conventional methods for fixing various enzymes can be employed. For instance, employable is a method of using a carrier of CNBr-activated Sepharose 4B (produced by Pharmacia Fine Chemicals Co.) or the like. To actually purify human TPO by the use of the fixed anti-human TPO antibody, the fixed anti-human TPO antibody is filled into a column and a human TPO-containing liquid is passed through the column. By this operation, a large amount of human TPO is adsorbed onto the carrier in the column. As the solvent for elution, for example, usable are glycine-HCl buffer (pH 2.5), sodium chloride solution, propionic acid, dioxane, ethylene glycol, chaotropic salt, guanidine hydrochloride, urea, etc. By the elution with such a solvent, human TPO having a high purity is eluted.

The antibody of the present invention can be used for determining human TPO by immunochemical quantitative assay, especially by enzyme-immunoassay to be conducted by a solid phase sandwich process.

An advantage of monoclonal antibodies is that they can be produced by hybridoma cells in a medium which does not contain any other immunoglobulin molecules. Monoclonal antibodies can be prepared from culture supernatants of hybridoma cells or from mouse ascites induced by intraperitoneal injection of hybridoma cells. The hybridoma technique originally disclosed by Kohler and Milstein (Eur. J. Immunol. 6:511-519, 1976) can be used broadly for the formation of hybrid cell lines which are possessed of high level monoclonal antibodies for a number of specific antigens.

To isolate the intended antibody from the thusobtained antibody-containing material, such as anti-serum, etc., by purifying it, one or more steps (affinity chromatography such as protein A affinity chromatography, protein G affinity chromatography, Avid gel chromatography, anti-immunoglobulin-fixed gel chromatography, etc.; as well chromatography, anion-exchange cation-exchange affinity chromatography, lectin chromatography, interaction hydrophobic chromatography, adsorption chromatography, gel permeation chromatography, reversed phase chromatography, hydroxyl-apatite chromatography, chelating chromatography, metal fluoro-apatite chromatography, point isoelectric chromatography, partitioning electrophoresis, isoelectric point electrophoresis, etc.) which are generally employed for purification of protein Apart from these, a method of antigen can be combined. affinity purification can also be employed, in which a gel carrier or membrane, to which has been chemically bonded a human TPO protein itself or a peptide that contains the region of the antigen or a part of the region or, that is, a molecule capable of recognizing the intended antibody, is prepared, an antibody-containing material is added thereto so that the intended antibody is made to be adsorbed onto the carrier or membrane, and the thus-adsorbed antibody is then eluted and recovered under suitable conditions.

The present invention also allows use of endogenous DNA sequences encoding a TPO polypeptide for the production of large quantities of polypeptide products. For example, in vitro and ex vivo homologous recombination procedures may be employed to transform host cells to provide for expression or enhanced expression of polypeptides. Preferred host cells include human cells (e.g., liver, bone marrow and the like) wherein a promoter or enhancer sequence is inserted employing flanking sequences that are homologous to a target region in a cellular genome with the result that TPO polypeptide expression is accomplished or enhanced. See, e.g., U.S. Letters Patent 5,272,071, published PCT WO 90/14092, WO 91/06666 and WO 91/09955.

Also provided by the invention are processes for producing a TPO polypeptide as described above comprising the steps of expressing a polypeptide encoded by a DNA of the invention in a suitable host, and isolating said TPO polypeptide. Where the TPO polypeptide expressed is a Met<sup>-2</sup>-Lys<sup>-1</sup> polypeptide, such processes can further include the step of cleaving Met<sup>-2</sup>-Lys<sup>-1</sup> from said isolated TPO polypeptide.

Also provided are methods for producing TPO polypeptides having [Gly<sup>-1</sup>] structure comprising the steps of introducing into a suitable host cell a DNA encoding a glutathione-S-transferase (GST) polypeptide 5' to TPO polypeptide encoding sequences, the GST and TPO polypeptide encoding sequences separated by DNA encoding a thrombin recognition polypeptide, isolating the GST-TPO expression

product and treating the expressed polypeptide with thrombin to remove GST amino acids. The resulting TPO polypeptides have a [Gly-1] structure.

The following describes the present invention in detail.

(A) Purification of rat TPO, analysis of partial amino acid sequences of purified rat TPO and analysis of biological characteristics of purified rat TPO

The inventors of the present invention have firstly attempted to purify a protein (rat TPO) which has an activity to enhance proliferation and differentiation of rat CFU-MK. In this purification study, many trial and error attempts were made on the purification procedures, such as selection of various natural supply sources, selection of gels for chromatographies and separation modes. As the result, the present inventors have succeeded in purifying a protein having a TPO activity from blood plasma of thrombocytopenic rats induced by X-ray or  $\gamma$ -ray irradiation, making use of the TPO activity as a marker based on a rat CFU-MK assay which will be described later in <Reference Example>, and in determining partial amino acid sequences of the purified protein <Examples 1 and 2>.

Biological characteristics of the plasma-derived rat TPO were also examined <Example 3>.

An outline of the procedures from the purification of rat TPO to the determination of partial amino acid sequences of the purified protein is shown in the following.

(i) A blood plasma sample was prepared from about 1,100 thrombocytopenic rats induced by X-ray or  $\gamma$ -ray irradiation and subjected to a Sephadex G-25 chromatography, an anion exchange chromatography (Q-Sepharose FF) and a

lectin chromatography (WGA-Agarose) in that order, thereby obtaining a WGA-Agarose-adsorbed TPO-active fraction.

- (ii) Next, the thus obtained WGA-Agarose-adsorbed TPO-active fraction was subjected to a triazine dye affinity chromatography (TSK AF-BLUE 650MH), a hydrophobic interaction chromatography (Phenyl Sepharose 6 FF/LS) and a gel filtration chromatography (Sephacryl S-200 HR) in that order. Since the TPO activity was divided into 4 peaks (F1 as the highest molecular weight fraction, followed by F2, F3 and F4) by the Sephacryl S-200 HR gel filtration, each of the TPO-active fractions F2 and F3 was concentrated to obtain a high molecular weight TPO sample F2 and a low molecular weight TPO sample F3 which were used separately in the subsequent purification steps.
- (iii) The low molecular weight TPO sample F3 was subjected to a preparative reverse phase chromatography (YMC-Pack PROTEIN-RP), a reverse phase chromatography (YMC-Pack CN-AP) and another reverse phase chromatography (Capcell Pack C1) in that order. When the thus obtained TPO-active fraction was applied to Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) and the TPO-active substance was extracted from the gel, the presence of the TPO activity was confirmed in a band corresponding to apparent molecular weights of about 17,000 to 19,000 under a non-reducing condition.
- (iv) In consequence, all TPO-active fractions were applied to SDS-PAGE under a non-reducing condition and transferred on a PVDF membrane. By carrying out systematic limited enzymatic hydrolysis of the protein on the PVDF membrane into peptide fragments, partial amino acid sequences of the rat TPO protein were determined. Based on the amino acid sequence information of two peptide fragments, cloning of rat TPO gene was performed.

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(v) Separately from this, the high molecular weight TPO sample F2 obtained by the Sephacryl S-200 HR was subjected to purification in the same manner as in the case of the low molecular weight TPO sample F3. When a TPO-active fraction obtained by the final step reverse phase chromatography (Capcell Pack C1) was applied to SDS-PAGE and the TPO-active substance was extracted from the gel, the presence of the TPO activity was confirmed in a band corresponding to apparent molecular weights of about 17,000 to 22,000 under a non-reducing condition.

# (B) Specialization of rat TPO producing cells, preparation of mRNA and construction of rat cDNA library

Since the thus purified rat TPO has been derived from blood plasma, it was necessary to screen organs or cells as the source of mRNA for use in the cDNA cloning. In consequence, TPO activities in various organs and cell culture supernatants were screened based on the biochemical and biological properties of the rat plasma-derived TPO. As the result, TPO activities almost equal to the rat plasma-derived TPO were found in the culture supernatant of rat hepatocyte-derived cell lines McA-RH8994, HTC and H4-II-E, as well as in the culture supernatant of rat primary hepatocytes <Example 4>.

On the other hand, the expression vector pEF18S was constructed from 2 expression vectors pME18S and pEFBOS <Example 5>. Construction of this vector has rendered possible easy cloning of cDNA using a high efficiency expression vector having multiple cloning sites which can be used for the integration of inserts.

In addition to the above expression vector, pUC and pBR based plasmid vectors and  $\lambda$  based phage vectors are mainly used for the construction of cDNA libraries.

The McA-RH8994 cells were cultured, homogenized by adding guanidine thiocyanate solution and then subjected to CsCI density gradient centrifugation to obtain total RNA <Example 6>.

Preparation of RNA can also be made by a hot phenol method, an acid guanidium phenol chloroform method and the like.

After purifying poly (A)+ RNA from the total RNA using oligo dT-immobilized latex particles, first strand cDNA was synthesized by the action of reverse transcriptase using oligo dT as a primer to which a restriction enzyme Notl recognition sequence has been added, followed by the synthesis of 2nd strand cDNA using RNase H and E.coli DNA polymerase I. An EcoRl adaptor was added to the thus obtained double-strand cDNA, the resulting cDNA was ligated with a mammalian cell expression vector pEF18S constructed in Example 5, which has been digested with Notl and EcoRl and then the thus ligated cDNA was transformed into competent cells of an E. coli strain DH5 to construct a cDNA library <Example 7>.

## (C) Preparation (cloning) of rat TPO cDNA fragment by PCR

A DNA sequence was deduced from the partial amino acid sequence of the rat TPO which has been purified from rat blood plasma, in order to synthesize degenerate primers to be used in the polymerase chain reaction (PCR). Primers to be used may also be obtained based on an amino acid sequence other than the position of the primers used herein. Highly degenerate primers may also be used without employing inosine. In addition, primers with reduced degeneracy can be designed making use of a codon having high usage in rat (Wada et al., *Nucleic Acids Res.*, vol.18, p.2367-2411, 1990).

When plasmid DNA was extracted from entire portion of the cDNA library prepared above and PCR was

carried out using the extracted DNA as a template, a band of about 330 bp was detected which was subsequently determined by nucleotide sequence analysis as a DNA fragment (A1 fragment) that encodes a portion of the rat TPO <Example 8>.

# (D) Screening of rat TPO cDNA by PCR, sequence of rat TPO cDNA and confirmation of TPO activity

The cDNA library prepared above was divided into pools, each containing about 10,000 clones, and plasmid DNA was extracted from each of 100 pools. When PCR was carried out using each pool of plasmid DNA as a template and primers newly synthesized based on the nucleotide sequence of the A1 fragment, bands considered to be specific were detected in 3 pools. One of these 3 pools was divided into sub-pools, each containing about 900 clones, and plasmid DNA was purified from 100 sub-pools to carry out PCR in the same manner. As the result, a specific band was detected in 3 sub-pools. One of these pools was divided into sub-pools, each containing 40 clones, and finally each clone was screened by PCR in the same manner. As the result, a clone pEF18S-A2α which seemed to encode the rat TPO cDNA was isolated <Examples 9 and 10>.

When nucleotide sequence of this clone was analyzed, it was revealed that it encodes the partial amino acid sequence of the protein purified from rat blood plasma, thus confirming a strong possibility that this clone contains the rat TPO cDNA <Example 10>.

When plasmid DNA was purified from the clone pEF18S-A2 $\alpha$  obtained above and transfected into COS 1 cells, TPO activity was found in the culture supernatant of the transfected cells. As the result, it was confirmed that the clone pEF18S-A2 $\alpha$  contains a cDNA which encodes the rat TPO <Example 11>.

## (E) Detection of TPO mRNA in various rat tissues

TPO mRNA expression in rat tissues was analyzed by PCR, and specific expression was detected in the brain, the liver, the small intestines and the kidney <Example 12>.

## (F) Construction of human cDNA library

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Based on the results of Examples 4 and 12, the liver was selected as the starting tissue for the cloning of human TPO cDNA. In consequence, a cDNA library was constructed using a commercial mRNA derived from normal human liver. Using pEF-18S as a vector like the case of the rat library, cDNA was synthesized by the same procedure as the case of rat to obtain a directionally cloned cDNA library by making use of restriction enzymes *Not*I and *EcoRI*. The library prepared by introducing vector-ligated cDNA into *E. coli* DH5 contained about 1,200,000 clones <Example 13>.

# (G) Preparation (cloning) of human TPO cDNA fragment by PCR

Several primers for PCR were synthesized based on the nucleotide sequence of the clone pEF18S-A2 $\alpha$  which encodes the rat TPO cDNA. When cDNA was synthesized using a commercial mRNA derived from normal human liver and PCR was carried out using these primers and cDNA as a template, a band of around 620 bp was observed. When the nucleotide sequence was analyzed, it was revealed that this clone contains a DNA fragment which has a homology of about 86% with the rat TPO cDNA, thus confirming a strong possibility that this is a part of a gene which encodes human TPO <Example 14>.

(H) Screening of human TPO cDNA by PCR, sequence of human TPO cDNA and confirmation of TPO activity

The human cDNA library prepared above was amplified, divided into pools, each containing about 100,000 clones, and plasmid DNA was extracted from each of 90 pools. When PCR was carried out using each pool of plasmid DNA molecules as a template and primers newly synthesized based on the nucleotide sequence of the human TPO fragment obtained in Example 14, possible bands were detected in 3 pools. One of these pools was divided into sub-pools, each containing 5,000 clones, and plasmid DNA was purified from each of 90 sub-pools. When PCR was carried out using each as a template in the same manner, pool of plasmid DNA possible bands were detected in 5 sub-pools. When one of these pools was divided into sub-pools, each containing 250 clones, and plasmid DNA was purified from each of 90 subpools and subjected to PCR, possible bands were detected in 3 When one of these pools was divided into subpools, each containing 30 clones, and plasmid DNA was purified from each of 90 sub-pools and subjected to PCR, possible bands were detected in 3 sub-pools. Thereafter, 90 colonies were isolated from one of these sub-pools and plasmid DNA purified from each of the colonies was subjected to PCR to finally obtain a clone named HL34 <Example 15>.

When nucleotide sequence of plasmid DNA in this clone was analyzed, it was revealed that this clone contains a cDNA which has about 84% homology with the rat TPO cDNA nucleotide sequence <Example 16>.

When the cloned plasmid DNA was purified and transfected into COS 1 cells, TPO activity was found in the culture supernatant of the transfected cells. As the result, it was confirmed that this plasmid clone contains the cDNA which encodes the human TPO <Example 17>.

However, this cDNA seemed to be an artificial product of the cloning, because no termination codon was found in this clone and a poly A tail-like sequence was found on its

3'-end. In consequence, an expression vector was constructed which encodes the amino acid sequence excluding a corresponding portion to the poly A tail-like sequence. When the thus constructed vector was expressed in COS 1 cells, TPO activity was found in the resulting culture supernatant <Example 18>.

DNA fragment in the 3'-end region of the human TPO was obtained by PCR so as to analyze the structure of a full-length cDNA.

The determination of the nucleotide sequence of this fragment revealed that it partly overlaps with cDNA carried by the clone HL34 obtained in Example 15. It was also expected that a full length human TPO cDNA may contain an open reading frame thereof and code for a protein consisting of 353 amino acids. Thus, it was suggested that the human TPO consisted of 353 amino acids including a signal sequence of 21 amino acids <Example 19>.

In addition to the above cloning method, cloning of human TPO cDNA can be attained by colony hybridization or plaque hybridization using a rat TPO cDNA fragment as a probe, making use of a library constructed using a pUC or pBR based plasmid vector, a  $\lambda$  phage based vector or the like. In designing a degenerate probe; inosine may be used to decrease the degree of degeneracy. When an assay method which can detect TPO activity in a specific manner or with a high sensitivity is available, it is possible to carry out expression cloning making use of an expression library as used in the present invention.

However, since the content of human TPO-encoding RNA seems to be extremely low in the normal human liver as will be described later in Example 15 (the content calculated from the result of this Example was in a ratio of one per three million), the hybridization screening in which a synthesized oligonucleotide or a rat or a human TPO cDNA fragment is used as a probe will be extremely difficult to effect, because the

number of clones or plaques to be treated becomes bulky and sensitivity and specificity of the hybridization method are inferior to those of the PCR method. As a matter of fact, the inventors of the present invention have carried out colony hybridization of two million clones in a normal human liver cDNA library prepared in Example 13 using a rat TPO cDNA fragment as a probe, but a human TPO cDNA clone was not able to be obtained.

## (1) Reconstruction of human normal liver-derived cDNA

Since the clone HL34 obtained in Example 15 seemed to contain an incomplete cDNA, a cDNA library was reconstructed using a commercial normal human liver-derived poly (A)<sup>+</sup> RNA preparation to obtain a complete human TPO cDNA. The library (hTPO-F1) prepared by introducing vector-ligated cDNA into E. coli DH5 contained 1.0 x 10<sup>6</sup> transformants <Example 20>.

# (J) Screening of TPO cDNA clone, sequence determination and expression of human TPO cDNA, and confirmation of TPO activity

Primers for PCR were synthesized based on the nucleotide sequence (SEQ ID NO: 3) of a partial cDNA obtained in Example 14 and the nucleotide sequence (SEQ ID NO: 196) of a complete cDNA of human TPO predicted in Example 19.

The human liver cDNA library (hTPO-F1) constructed in Example 20 was divided into 3 pools (pool # 1 - 3). PCR was carried out using the plasmid DNA prepared from each pool as a template and the synthesized primers. As the result, a DNA fragment having the expected size was amplified when the plasmid DNA prepared from the pool # 3 was used. The # 3 pool was then divided into subpools, each containing 15,000 transformants, and screening using PCR was carried out as above. As the result, amplification of DNA having the

expected size was found in 6 of the 90 pools. When one of these positive pools was divided into subpools, each containing 1,000 clones, and plasmid DNA was extracted and PCR was carried out in the same manner, DNA amplification was not observed. It was considered to be caused by a low recovery of the plasmid DNA due to poorer growth of the clone of interest than the other clones. Therefore, the original # 3 pool was spread on 100 LB plates in such an inoculum size that 4,100 colonies grew on each LB plate, and a replica plate was prepared from each of the thus inoculated plates. When PCR using these DNAs extracted from colonies grown on the plate was carried out in the same manner as described above, amplification of a band expected was observed in one of 100 subpools.

Two replica filters were prepared from the plate of this subpool, and colony hybridization was carried out using the labeled probe(the EcoRI/BamHI fragment of plasmid pEF18S-HL34). As the result, a single signal considered to be positive was observed. The colonies were collected from the original plate and inoculated again onto an LB plate. samples were prepared from a total of 50 colonies grown on the plate and were subjected to PCR to finally obtain a clone named pHTF1 <Example 21>. In screening of cDNA clone, methods such as hybridization, PCR, and expression cloning were mainly used and combination of these methods increased efficiency and sensitivity of the screening, or decreased labor for it. When the nucleotide sequence of the thus obtained clone pHTF1 was determined, it was revealed that the clone pHTF1 had an open reading frame and the amino acid sequence of a protein considered to be encoded by the open reading frame completely coincided with the deduced amino acid sequence (SEQ ID NO: 6) of human TPO. The nucleotide sequence was different from the deduced one (SEQ ID NO: 196) at 3 positions, but these differences did not cause amino acid exchange. It is confirmed that human TPO protein comprises 353 amino acid residues having a 21 amino acid signal sequence. When the plasmid DNA was prepared from the thus obtained clone pHTF1 and transfected into COS1 cells, TPO activity was found in the culture supernatant <Example 23>.

(K) Screening of human TPO chromosomal DNA by means of plaque hybridization, sequence determination and expression of human TPO chromosomal DNA and confirmation of TPO activity

A genomic library given from Prof. T. Yamamoto at the Gene Research Center, Tohoku University was inoculated onto 18 NZYM plates in such an inoculum size that one plate contained 30,000 phage particles, and two replica filters were prepared from each of the thus prepared 18 plates. A human TPO cDNA fragment (base position numbers 178 to 1,025 in SEQ ID NO: 7) was amplified by PCR and purified. Using the purified fragment labeled with 32P as a probe, plaque hybridization was carried out. As the result, 13 positive signals were obtained. Plaques were collected from the original plates and inoculated again onto NZYM plates in such an inoculum size that 1,000 plaques were formed on each plate. Two replica filters were prepared from each of the resulting plates to carry out plaque hybridization under the same conditions described above. As the result, positive signals were detected on all filters of the 13 groups. A single plaque was recovered from each of the resulting plates to prepare phage DNA. Phage DNA samples thus prepared from the 13 clones were checked for the presence of the cDNA coding region by PCR. Five of the 13 clones seemed to contain entire amino acid coding region predicted from the cDNA.in consequence, one of these clones (clone \( \lambda HGT1 \)) was selected and analyzed by Southern blotting (using the aforementioned probe). Since a single band of about 10 kb was observed in the case of the HindIII digestion, DNA of the clone  $\lambda$ HGT1 was digested with HindIII and subjected to agarose gel electrophoresis. The 10 kb band was excised from the gel, purified, and subcloned into a cloning vector pUC13 to finally obtain a clone named pHGT1 <Example 24>.

When the nucleotide sequence of the thus obtained clone pHGT1 was determined, chromosomal DNA carried by the clone was found to contain an entire coding region of the protein predicted in Example 19, and nucleotide sequence of the coding region coincided completely with that of the predicted nucleotide sequence (SEQ ID NO: 196).

In addition, a region corresponding to the amino acid-encoding exon contained 4 introns and the nucleotide sequence was different from that of the complete cDNA carried by clone pHTF1 obtained in Example 21 (SEQ ID NO: 7).

When nucleotide sequences of 4 remaining clones among the 5 chromosomal DNA clones independently selected by the screening were determined, it was found that 2 of the 4 clones were identical to the clone pHGT1 and other 2 clones were the same as the clone pHGT1 except that they had a different nucleotide within 3' noncoding region as observed with the clone pHTF1 <Example 25>.

An EcoRI fragment in the thus obtained plasmid clone pHGT1 was ligated with an expression vector pEF18S to obtain human TPO expression plasmid pEFHGTE. When the plasmid DNA (pEFHGTE) was prepared and transfected into COS1 cells, TPO activity was found in the culture supernatant <Example 26>.

(L) Preparation of human TPO deletion derivatives, their expression in COS1 cells and confirmation of TPO activity

The results of Example 18 and 22 revealed that human TPO protein could exhibit its biological activity even after the removal of its carboxyl-terminal third. Thus, in order to further analyze the biologically active portions, deletion derivative experiments were performed. A series of expression plasmids were prepared by PCR using the DNA of the plasmid clone pHT1-231 obtained in Example 18 as a template and synthesized oligonucleotides as primers.

Expression plasmids which contained DNA coding for human TPO deletion derivatives lacking the carboxylterminal region of the TPO protein, that is, deletion derivatives coding for positions 1-211, 1-191, 1-171 and 1-163 amino acid sequence, were obtained. When the plasmid DNA was prepared from each of clones and transfected into COS1 cells, TPO activity was detected in each of the culture supernatant <Example 27>.

When derivatives having a series of deletion of C-terminal amino acid residues up to the 151 position and other derivatives having respective deletion of N-terminal side amino acid residues up to the 6, 7 and 12 position were designed and the activity after their expression in COS 1 cells was measured in order to analyze the TPO activity-bearing region further in detail, the activity became undetectable when N-terminal side amino acid residues were deleted up to the 7 position or C-terminal side amino acid residues were deleted up to the 151 position. <Examples 28 and 29>

Derivatives of a protein having its TPO biological activity can be obtained by modifying (deletion, substitution, insertion or addition) cDNA coding the protein. Methods such as PCR, site directed mutagenesis and chemical synthesis can be used for the modification.

(M) Expression of human TPO cDNA in CHO cell and purification of TPO.

An expression vector, pHTP1, was constructed which can express cDNA coding for a deduced amino acid sequence of human TPO as shown in SEQ ID NO: 6, in animal cells. <Example 30>

The TPO cDNA region in pHTP1 was used to construct a vector pDEF202-hTPO-P1 for its expression in CHO cell. <Example 31>

As a result of the transfection of the vector into CHO cells and subsequent selection of transfected cells, the transfected cells in which the expression vector carrying human TPO cDNA has been integrated into chromosomes of CHO cells were obtained. <Example 32>

In Example 32, large scale cultivation was carried out for a human TPO producing CHO cell line (CHO 28-30 cell, resistant to 25 nM MTX) which has been prepared by transfecting the human TPO expression plasmid pDEF202-hTPO-P1 into CHO cells. <Example 55>

From the culture supernatant 100 L, human TPO was purified. <Example 56>

Alternatively, human TPO was purified from the culture supernatant in Example 55 in a different manner. <Example 57>

# (N) Expression of human TPO cDNA in X63.6.5.3. cells and confirmation of the activity

Using the TPO cDNA region encoded in the plasmid pBLTEN prepared in Example 30, an expression vector BMCGSneo-hTPO-P1 was constructed for use in X63.6.5.3. cells. <Example 33>

When X63.6.5.3. cells were transfected with this vector, a transformant cell was obtained in which the human cDNA-encoding expression vector was integrated into its

chromosome. When this cell was cultured, TPO activity was detected in the culture supernatant. <Example 34>

(O) Large quantity expression of human TPO in COS 1 cells, and purification, molecular weight measurement and biological characteristics thereof

The expression vector pHTP1 prepared in Example 30 was transfected into COS 1 cells to obtain a large quantity (a total of about 40 liters) of culture supernatant containing the expressed product. <Example 35>.

Purification of TPO was carried out from about 7 liters of the COS 1 cell serum-free culture supernatant prepared in Example 35 containing the expression vector pHTP1-derived TPO. It was able to obtain high activity TPO through the steps of a hydrophobic interaction chromatography, a cation exchange column chromatography, a WGA column chromatography and a reverse phase column chromatography. <Example 36>

The TPO thus partially purified from the COS 1 cell culture supernatant was subjected to molecular weight measurement and biological characteristics analysis. <Examples 37 and 38>

## (P) Expression of human TPO in E. coli

A vector pGEX-2T/hT(1-174) was constructed for use in the expression of a fusion protein (to be referred to as "GST-TPO(1-174)") of glutathione-S-transferase and human TPO (amino acid residues of positions 1 to 174) in *E. coli.* In this case, a portion of the human TPO cDNA nucleotide sequence (about half the area of the 5'-side) was exchanged with *E. coli* preference codons. <Example 39>

GST-TPO(1-174) was expressed in *E. coli*, the resulting cells were disintegrated and then the GST-TPO(1-174) contained in the precipitation fraction was solubilized.

Next, as a result of the combination of various steps including examination of the TPO refolding conditions, examination of purification conditions (glutathione affinity column, cation exchange column and the like) and digestion of the GST protein region with thrombin, it was able to perform partial purification of a protein containing a TPO amino acid sequence as designed. It was confirmed that this protein shows TPO activity in the rat CFU-MK assay system. <Examples 40 and 41>

Also, a vector pCFM536/h6T(1-163) was constructed for use in the expression in *E. coli* of a mutation type human TPO protein (to be referred to as "h6T(1-163") in which the 1-position Ser residue and the 3-position Ala residue of human TPO (amino acid residues of positions 1 to 163) were respectively replaced by an Ala residue and a Val residue and, at the same time, a Lys residue and a Met residue were respectively added to the -1 and -2 positions. Entire portion of the human TPO cDNA nucleotide sequence (corresponding to 1 to 163 amino acid residues) carried by this vector was exchanged with *E. coli* preference codons. <Example 42>

The h6T(1-163) was expressed in *E. coli*, the resulting cells were lysed and then solubilization of h6T(1-163) contained in the precipitated fraction and conditions for the refolding of the protein were examined, thereby succeeding in partially purifying a protein containing a TPO amino acid sequence as designed. It was confirmed that the protein shows TPO activity in the rat CFU-MK assay system. <Examples 43 and 44>

In addition, a vector pCFM536/hMKT(1-163) was constructed for use in the expression in *E. coli* of a mutation type human TPO protein (to be referred to as "hMKT(1-163)") in which a Lys residue and a Met residue were respectively added to the -1 and -2 positions. The hMKT(1-163) was expressed in

E. coli in the same manner as described in Example 43, and the expression protein was subjected to SDS-PAGE, transferred on a PVDF membrane and then subjected to N-terminal amino acid sequence analysis, thereby confirming that this protein contains the designed amino acid sequence. <Example 52>

In addition, the vector pCFM536/hMKT(1-332) for expression in *E. coli* of a mutation type human TPO protein in which a Lys residue is added at the position-1 and Met residue at the position-2 of human TPO (amino acid positions 1 to 332) (referred to as "hMKT (1-332)") respectively was constructed. The hMKT (1-332) was expressed within *E. coli* in the same manner as in Example 42, and its expression was confirmed by Western blotting using the anti-human TPO peptide antibody prepared in Example 45 set forth below. <Example 66>

## (Q) Preparation of anti-TPO peptide antibody and anti-TPO peptide antibody column

Rabbit polyclonal anti-TPO peptide antibodies were prepared by synthesized peptides corresponding to three partial regions of the rat TPO amino acid sequence determined in Example 10. It was confirmed that these antibodies can recognize rat and human TPO molecules. Furthermore, peptides corresponding to 6 partial regions in the amino acid sequence of human TPO shown in SEQ ID NO: 6 (or SEQ ID NO: 7) were synthesized and then employed to prepare rabbit polyclonal anti TPO peptide antibodies. The resultant antibodies were confirmed to recognize human TPO. <Example 45>

It is possible to purify TPO by an affinity column chromatography using a column packed with certain molecules having binding affinity for TPO, such as anti-TPO antibodies, TPO receptors and the like, are bound. In consequence, an anti-TPO antibody column was firstly prepared by binding the anti-TPO peptide antibody obtained in Example 45. <Example 46>

(R) Purification of human TPO expressed in COS 1 cells making use of anti-TPO peptide antibody column, and molecular weight and biological characteristics thereof

Using a culture supernatant from the transfection of COS 1 cells with the expression vector pHTP1 as a starting material, partially purified TPO was obtained and applied to the anti-TPO antibody column. Since the TPO activity was found in the adsorbed fraction, this fraction was further subjected to a reverse phase column chromatography to purify the protein and examine its molecular weight and biological characteristics. <Example 47>

(S) Confirmation of the activity of partially purified sample of human TPO expressed in COS 1 cells

The TPO-active fraction purified in Example 36, namely the TPO sample purified up to the step of Capcell Pak CI 300A column from a culture supernatant obtained by transfecting COS 1 cells with the expression vector pHTP1, was checked for its biological activities to determine if it exerts a platelet number increasing action in the living body. <Example 48>

Also, a crude TPO fraction obtained by a cation exchange column from 33 liters of a culture supernatant prepared by transfecting COS 1 cells with the expression vector pHTP1 was checked for its biological activities to find that it increases platelet number in the body. <Example 49>

(T) Expression of human TPO chromosomal DNA in CHO cells and confirmation of the activity

A vector pDEF202-ghTPO was constructed for use in the expression of human TPO chromosome in CHO cells. <Example 50>

When this vector was introduced into CHO cells, a transformant cell was obtained in which the human TPO chromosomal DNA-encoding expression vector was integrated into its chromosome. When this cell clone was cultured, TPO activity was detected in the culture supernatant. <Example 51>

# (U) Partial purification and activity confirmation of mutation type human TPO expressed in *E. coli*

The mutation type human TPO derived from the human TPO nucleotide sequence-encoding clone, pCFM536/h6T(1-163), and expressed in *E. coli* was subjected to refolding using guanidine hydrochloride and glutathione to find that the thus obtained h6T(1-163) exerts a platelet number increasing action in the living body. <Example 53>

The mutation type human TPO derived from the human TPO nucleotide sequence-encoding clone, pCFM536/h6T(1-163), and expressed in *E. coli* was subjected to refolding using sodium N-lauroyl sarcosinate and copper sulfate and then to a cation exchange chromatography to find that the thus purified h6T(1-163) increases platelet number in the living body. <Example 54>

In addition, the refolding and purification of the mutation type human TPO, h6T (1-163) was conducted using another procedures. <Examples 60 and 61>

## (V) Expression of human TPO cDNA in insect cells and identification of TPO activity

A recombinant virus for the expression of human TPO in insect cells was prepared <Example 58> and expressed

in the insect cell Sf 21, and subsequently the TPO activity was identified in the culture supernatant. <Example 59>

# (W). Expression of human TPO (amino acid positions 1 to 163) in CHO cells and purification thereof

The vector pDEF202-hTPO163 for expression in CHO cells of the human TPO protein (referred to as "hTPO163") having amino acids 1 to 163 in the human TPO amino acid sequence shown in SEQ ID NO: 7 was constructed. The expression vector pDEF202-hTPO163 was transfected into CHO cells and thus-obtained hTPO163-producing CHO cell line was cultured in a large scale. From the supernatant of the culture hTPO163 was purified. <Examples 62 to 65>

## (X) Preparation of substitution derivatives of human TPO

A derivative (referred to as "N3/TPO") wherein Arg-25 and Glu-231 of human TPO have been substituted by Asn and Lys, respectively, was prepared as well as a derivative (referred to as "09/TPO") wherein His-33 has been replaced by Thr.

A plasmid coding for each derivative was transfected into COS7 cells which were then cultured. TPO activity was detected in the supernatant of the culture. <Example 67>

Derivatives wherein one amino acid of h6T(1-163) has been substituted by an other amino acid were prepared by using E. coli expression system. TPO activity was found in each derivative. <Example 94>

(Y) <u>Preparation of insertion or deletion derivatives of human IPO</u>

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An amino acid is inserted into or deleted from hTPO163 to prepare its insertion or deletion derivatives, and thereafter COS7 cells are transfected with plasmids encoding the resultant derivatives. In the supernatant of the COS7 cell cultures, the TPO activity was detected. <Example 68>

A method for the measurement of TPO activity (an in vitro assay system) used in the present invention is described in the following as "Reference Example".

# (Z) <u>Preparation and purification of anti-human TPO</u> antibodies

Polyclonal antibodies are prepared using peptide fragments of human TPO <Example 69> and subsequently utilized in Western blot analysis <Example 70> and in the construction of anti-TPO antibody affinity columns <Example 71>.

#### (AA) In vivo activity of human TPO

Purified human TPO is administered to mice with induced thrombocytopenia and changes in platelet counts are monitored versus a control group. <Example 72>, through <Example 79>. Pharmaceutical compositions are described as potential useful in treatment of thrombocytopenia. <Example 80> through <Example 89>.

## (BB) TPO activity as a function of MLP receptor binding

An assay is provided wherein TPO activity is defined in terms of specific binding interaction with the MpI receptor. <Example 90> through <Example 93>.

#### Reference Example

A. Rat megakaryocyte progenitor cell assay (rat CFU-MK assay) system (liquid culture system) WO 95/21919 - 55 - PCT/JP95/00208

Megakaryocytes incorporate and store serotonin in cytoplasmic dense granules through an active, energydependent process (Fedorko, Lab. Invest., vol.36, pp.310 - 320, 1977). This phenomenon can be observed already in small and mononuclear acetylcholinesterase-positive cells which are considered to be positioned between CFU-MK and recognizable megakaryocytes (Bricker and Zuckerman, Exp. Hematol., vol.12, p.672, 1984). The amount of serotonin incorporated increases in response to the increment of the size of megakaryocytes (Schick and Weinstein, J. Lab. Clin. Med., vol.98, pp.607 - 615, In addition, it is known that such phenomenon is specific for only megakaryocytic cells among bone marrow cells (Schick and Weinstein, J. Lab. Clin. Med., vol.98, pp.607 -615, 1981). In the present assay system, highly enriched rat CFU-MK cells (GpIIb/IIIa+ CFU-MK fraction which will be described later) are cultured in the presence of samples to be assayed, and incorporation of <sup>14</sup>C-serotonin (<sup>14</sup>C-hydroxy tryptamine creatine sulphate; 14C-5HT) into megakaryocytes grown from CFU-MK is measured.

Advantages of this assay system are that indirect influences of contaminated cells (for example, formation of Meg-CSF activity by contaminated cells stimulated by a certain substance other than the factor of interest, or formation of a certain factor by contaminated cells, which undergoes a combined action with the factor of interest) can be reduced, because the percentage of CFU-MK in cells of GpIIb/IIIa+ CFU-MK fraction is markedly high (see the following [Assay method]) while the number of contaminating cells is small. In addition, appropriate culture conditions can be maintained for a relatively long period, because the total number of cells seeded per one well is small. Another advantage is that a number of large-size mature megakaryocytes grown from CFU-MK in the presence of an

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active sample during a culture period can visually detected under a phase contrast microscope, thus rendering possible qualitative judgement of the presence and degree of the activity. Results of the qualitative judgement correspond well to the results of quantitative determination based on the incorporation of <sup>14</sup>C-serotonin. Therefore, reliability of the quantitative determination can be further improved by joint use of the qualitative judgement.

#### Assav method:

Highly enriched rat CFU-MK cells (GpIIb/IIIa+ CFU-MK fraction) used in the assay were prepared by slightly modifying the method of Miyazaki et al. (Exp. Hematol., vol.20, pp.855 - 861, 1992).

Briefly, femur and tibia are removed from Wister rats (male, 8 to 12 weeks of age) to prepare bone marrow cell suspension as described. A suspending medium (a solution consisting of 13.6 mM trisodium citrate, 11.1 mM glucose, 1 mM adenosine, 1 mM theophylline, 10 mM HEPES (pH 7.25), 0.5% bovine serum albumin (to be referred to as "BSA" hereinafter) (Path-O-Cyte 4; Seikagaku Kogyo Co., Ltd.) and Ca2+ and Mg2+free Hanks' balanced salt solution: to be referred to as "HATCH solution" hereinafter) which is a slightly modified version of the megakaryocyte separation medium reported by Levine and Fedoroko (Levine and Fedoroko, Blood, vol.50, pp.713 - 725, 1977) is used for the preparation of the bone marrow cell suspension. The thus prepared bone marrow cell suspension is layered over a Percoll discontinuous density gradient solution (density: 1.050 g/ml/1.063 g/ml/1.082 g/ml) which is prepared by diluting Percoll solution (manufactured by Pharmacia) with HATCH solution, and centrifuged at 20°c at After the centrifugation, cells 400 x g for 20 minutes. between the 1.063 g/ml and 1.082 g/ml density layers are recovered. After washing, the recovered cells are suspended in

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Iscove's modified Dulbecco culture medium (to be referred to as "IMDM culture medium" hereinafter) containing 10% fetal calf serum (to be referred to as "FCS" hereinafter), put in 100mm tissue culture plastic dishes and cultured at 37°C for 1 hour in a 5% CO<sub>2</sub> incubator. After incubation, nonadherent cells are recovered and cultured again in plastic dishes at 37 °C for 1 hour. Non adherent cells are suspended in HATCH solution and incubated for 1 hour on 100-mm bacteriological petri dishes to which a mouse monoclonal anti-rat platelet Gpllb/Illa antibody, P55 antibody (Miyazaki et al., Thromb. Res., vol.59, pp.941 - 953, 1990) has previously been adsorbed. After incubation, nonadherent cells are removed by thorough washing with HATCH solution, and the remaining cells adsorbed to the immobilized P55 antibody are detached by In general, 3-4 x 10<sup>5</sup> cells are pipetting and collected. obtained from one rat. The thus obtained cell fraction contains highly enriched rat CFU-MK (to be referred to as "Gpllb/Illa+ CFU-MK fraction" hereinafter), and it is known that this cell fraction contains about 5 to 10% of CFU-MK based on the measurement by a colony assay system in the presence of a saturated concentration of rat IL-3. A hybridoma capable of producing the aforementioned P55 antibody has been deposited under the deposit No. FERM BP-4563 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 14, 1994.

Next, cells of the thus obtained GpIlb/IIIa<sup>+</sup> CFU-MK fraction are suspended in IMDM culture medium containing 10% FCS and dispensed in portions of  $10^4$  cells per well of a 96 well tissue culture plate. Each well is further supplied with a standard sample (will be described later in detail), or a sample to be assayed, thereby adjusting the final medium volume to 200  $\mu$ I/well. The thus prepared plate is put in a CO<sub>2</sub> incubator and incubated for 4 days at 37 °C. On 4th days of incubation,

0.1 µCi (3.7 KBq) of 14 C-serotonin is added to each well 3 hours before the completion of culturing, and the final incubation is continued at 37°C. After 3-hr incubation, the plate is centrifuged at 1,000 rpm for 3 minutes, and the resulting supernatant fluid is removed by suction. A 200 μl portion of PBS containing 0.05% EDTA is added to each well, and the plate is centrifuged to wash the plate by removing the resulting supernatant fluid. This washing step is repeated once again. A 200 µl portion of 2% Triton X-100 is added to the thus obtained pellet of cells in each well, and the plate is shaken on a plate mixer for approximately 5 to 10 minutes to A 150 µl portion of the thus lyse the cells thoroughly. obtained cell lysate is transferred into a commercial capshaped solid scintillator (Ready Cap; manufactured by Beckman) which is subsequently left overnight at 50°C in an oven to dry the lysate. On the next day, the Ready Cap is put into a glass vial to measure radioactivity of 14C by a liquid scintillation counter.

In this instance, almost the same results as those of the <sup>14</sup>C-serotonin incorporation assay can be obtained when acetylcholinesterase activity in the aforementioned cell lysate is measured according to the procedure of Ishibashi and Burstein (Blood, vol.67, pp.1512 - 1514, 1986).

#### Standard samples:

Firstly, blood plasma of thrombocytopenic rats was prepared in the following manner, for use in the preparation of standard samples.

Normal male Wistar rats (7 to 8 weeks old) were rendered thrombocytopenic by intravenous administration of the aforementioned P55 antibody with a dose of 0.5 mg per animal, twice with an interval of about 24 hours. About 24 hours after the second administration, blood was collected from the abdominal aorta under ether anesthesia, using a 10 ml

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capacity syringe in which 1 ml of 3.8% (v/v) trisodium citrate solution has been included as an anti-coagulation agent. The thus collected blood sample was dispensed into plastic centrifugation tubes and centrifuged at 1,200 x g for 10 minutes to recover blood plasma fraction. The thus recovered blood plasma fraction was again centrifuged at 1,200 x g for 10 minutes, and the resulting blood plasma fraction was recovered, taking care to avoid the pellet consisting of cells. platelets and the like, and pooled. (The blood plasma obtained in such a manner is referred to as "TRP" hereinafter.) treated TRP (standard sample C), WGA-Agarose column active fraction (standard sample W) or Phenyl Sepharose 6 FF/LS column active fraction (standard sample P) obtained from TRP in accordance with the procedure of Example 1 were dialyzed extensively against a sufficient volume of IMDM culture medium and used as assay standards.

In the rat TPO purification process described in Example 1, the standard sample C was used in the early stage, but was changed to the standard sample W halfway thereafter and then to the standard sample P. Specific activity of the standard sample C was defined tentatively as 1, and relative activities of the standard samples W and P were calculated based on the definition. Relative activity of each sample to be assayed was determined by comparing the dose-response curve of the standard sample to those of the samples to be assayed, and relative activity of the sample to be assayed was defined as n where its activity is n times higher than that of the standard sample C.

### B. Colony assay system

In this assay system, bone marrow cells are cultured in a semi-solid culture medium in the presence of a sample to be assayed, and the Meg-CSF activity is measured by

counting the number of megakaryocyte colonies formed by the proliferation and differentiation of CFU-MK.

#### Assav method:

## (a) In the case of the use of unseparated rat bone marrow cells and the like

A 1 ml final volume of IMDM culture medium containing un-separated rat bone marrow cells, cells obtained in each separation step of Gpllb/Illa+ CFU-MK of the aforementioned assay system A, or cells of GpIlb/Illa+ CFU-MK fraction, and 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 0.3% agar (AGAR NOBLE, manufactured by DIFCO) was put in a 35-mm tissue culture plastic dish and, after solidification at room temperature. cultured at 37°C in a CO2 incubator. In general, the number of cells per one dish is adjusted to 2-4  $\times$   $10^5$  in the case of unseparated bone marrow cells, 2-5 x 104 in the case of cells at the step of the Percoll density gradient or of the adherence depletion, or 0.5-2 x 103 in the case of cells of Gpllb/Illa+ CFU-MK fraction. After 6 to 7 days of the culture, the agar disks are detached from the dishes and placed on glass slides (76 mm x 52 mm). To dry each agar disk, a piece of 50-\_m nylon mesh and filter paper are placed, in that order, over the gel. The thus dried agar slides are heat-fixed for 5 minutes at 50 °C on a hot plate and soaked for 2 to 4 hours in an acetylcholinesterase staining solution prepared according the procedure of Jackson (Blood, vol.42, pp.413 - 421, 1973). When sufficient staining of megakaryocytes is confirmed, the agar slides are washed with water, dried, subjected to poststaining with Harris' hematoxylin solution for 30 seconds, washed with water and then air-dried. Megakaryocyte colonies are defined as 3 or more tightly grouped acetylcholinesterasepositive cells.

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# (b) In the case of the use of unseparated mouse bone marrow cells

The colony assay can be performed in the same manner as the above procedure (a) using 2-4 x  $10^5$  cells per one dish.

# (c) In the case of the use of human bone marrow cells or human cord blood cells

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Human bone marrow cells or human cord blood cells may be used as such, or as CFU-MK fractions enriched by the following manner.

Firstly, bone marrow fluid or cord blood is layered over Lymphoprep (manufactured by Daiichi Kagaku Co., Ltd.) and centrifuged, and the resulting interface leucocyte fraction is recovered. From the thus recovered cell fraction, cells to which biotinylated monoclonal antibodies specific for human surface antigens (CD2, CD11c and CD19) bind are removed with The cells removed by the avidin-linked magnetic beads. magnetic beads method are mainly B cells. T cells. macrophages and a part of granulocytes. The remaining cells are stained with FITC-labeled anti-CD34 antibody and PElabeled anti-HLA-DR antibody, and then a CD34- and HLA-DRpositive fraction is recovered using a cell sorter (for example, ELITE manufactured by COULTER). CFU-Mil cells are concentrated in this fraction (to be referred to as "CD34+DR+ CFU-MK fraction" hereinafter). Colony assay of human cells can be carried out in the same manner as the aforementioned case of the use of rat bone marrow cells, except that 3-5 x 103 cells of the CD34+DR+ CFU-MK fraction are used in one dish and a mixture of 12.5% human AB plasma and 12.5% FCS is used instead of 10% FCS. The culturing period for the formation of megakaryocyte colonies is 12 to 14 days. For the detection of human megakaryocytes, immunological staining of megakaryocytes is carried out by an alkaline phosphatase-anti-alkaline phosphatase antibody method with a mouse monoclonal antibody specific for a megakaryocyte surface antigen Gpllb/Illa (for example, Teramura et al., Exp. Hematol., vol.16, pp.843 - 848, 1988), and colonies each consisting of 3 or more megakaryocytes are counted.

# C. Assay system with a human megakaryoblast cell line (M-07e assay)

M-07e cells, a human megakaryoblast cell line, proliferate in response to GM-CSF, IL-3, SCF, IL-2 and the like (Avanzi et al., J. Cell. Physiol., vol.145, pp.458 - 464, 1990). Since these cells also respond to TPO, they are applicable to a substitutive assay system for the rat CFU-MK assay system.

#### Assav method:

M-07e cells maintained in the presence of GM-CSF are recovered, washed thoroughly and then suspended in IMDM culture medium containing 10% FCS. The resulting M-07e cell suspension is dispensed in portions of  $10^4$  cells into wells of a 96 well tissue culture plate, and each well is further supplied with a standard sample or a sample to be assayed, thereby adjusting the final volume to 200  $\mu$ l/well. The thus prepared plate is put in a 5% CO<sub>2</sub> incubator and incubated for 3 days at 37 °C. After 3 days of culture, 1  $\mu$ Ci (37 KBq) of  $^3$ H-thymidine is added to each well 4 hours before the completion of culturing. After completion of the culturing, the cells are collected on a glass fiber filter using a cell harvester to measure  $^3$ H radioactivity with a liquid scintillation counter (for example, Beta Plate manufactured by Pharmacia).

D. An assay system (Ba/F3 assay) in which a mouse pro-B cell line is used

Mouse pro-B cell line Ba/F3 is known as a cell line which proliferates in response to IL3, IL4 and the like (Palacios et al., Cell, vol.41, pp.727 - 734). Since its substrain BF-TE22 is capable of cell proliferation in response to not only IL3 and IL4 but also TPO, it can be applied to an assay system as a substitute for the rat CFU-MK assay or the human megakaryoblastic cell line-aided assay (M-07e assay) is as Briefly, BF-TE22 cells growing in Iscove's modified DME medium(IMDM:GIBCO) in the presence of 1 ng/ml of mouse IL-3 are recovered, washed three times with IMDM and suspended in IMDM medium containing 10% FCS. Next, the cell suspension is dispensed into wells of a 96 well tissue culture plate of a density of 1 x 104 cells per well, each well is further supplemented with a standard TPO solution or a sample to be tested and adjusted to a final volume of 200 µl/well. The resulting plate is incubated for 2 to 3 days in a 5% CO2 On the 2nd or 3rd day, 1  $\mu \text{Ci}$  (37 KBq) of  $^3\text{H}$ thymidine is added to each well and the culturing is continued for additional 4 hours. Finally, the cells are collected on a glass fiber filter using a cell harvester to measure 3H radioactivity with a liquid scintillation counter. In this assay system, almost all of cells cultured in media lacking TPO activity die and therefore do not incorporate <sup>3</sup>H-thymidine. However, cells cultured in media containing TPO activity show active proliferation in a TPO concentration-dependent manner and incorporate 3H-thymidine. In addition, results of this assay are parallel to those of the M-07e and CFU-MK assays.

The following examples are provided to further illustrate the present invention.

#### EXAMPLE 1-1

Purification of rat TPO from blood plasma of thrombocytopenic rats induced by anti-platelet antibody administration

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Preparation of blood plasma of thrombocytopenic rats induced by anti-platelet antibody administration

TRP was prepared as the purification source from about 1,000 rats in accordance with the procedure described in the aforementioned <Reference Example> A, rat megakaryocyte precursor cell (CFU-MK) assay system.

## Purification of rat TPO from TRP

Initially, purification was carried out using TRP of about 1,000 rats on an assumption that TPO content in TRP would be one per three million at the most, and a little less than 1 pmole of partially purified rat TPO was obtained. Though it is difficult in general, an attempt was made to analyze its partial amino acid sequences and three partial amino acid sequences were obtained but with a low accuracy. These sequences coincided with or are close to the amino acid sequence of a serine protease inhibitor (SPI) produced in the liver. It was unclear from these results whether TPO has a structure similar to that of the serine protease inhibitor or the sequences are derived from those of contaminated proteins other than TPO. Using these uncertain sequences, cloning of TPO gene from a rat cDNA library was carried, but potential TPO-encoding genes could not be obtained. Thereafter. intensive studies were carried out based on an assumption that such a failure was due to low purity and insufficient amount of the sample analyzed. In order to obtain a purified final sample necessary for the amino acid analysis, purification was carried out in accordance with a process described in the following <Example 1-2>. Surprisingly, it was estimated from results of <Example 1-2> that the TPO content in TRP or XRP (will be described below) was such a extremely small amount as one per one hundred million to one billion of total plasma

proteins, so that its complete purification was extremely difficult.

## EXAMPLE 1-2

Purification of rat TPO from blood plasma of thrombocytopenic rats induced by total body X-ray or  $\gamma$ -ray irradiation [Preparation of blood plasma of thrombocytopenic rats induced by total body X-ray or  $\gamma$ -ray irradiation]

Normal male Wister rats (7 to 8 weeks old) were rendered thrombocytopenic by whole body irradiation with X-rays or γ-rays at a sublethal dose (6 to 7 Gy). Blood was collected from the rats on the 14th day after irradiation and a blood plasma fraction (to be referred to as "XRP" hereinafter) was prepared in the same manner as the aforementioned procedure for the preparation of TRP.

In this case, XRP (ca. 8 L) prepared from a total of about 1,100 rats was used as the purification source.

## Purification of rat TPO from XRP

The blood plasma sample of about 1,100 rats was too large to process at one time because its total protein content reached 493,000 mg. In consequence, the blood plasma sample was divided into 11 lots, each corresponding to about 100 rats, in the following purification steps (1) to (4). In the subsequent purification steps (5) to (7), the sample was divided into 6 batches. In and after the purification step (8), a sample crudely purified from the total blood plasma of about 1,100 rats was used. In the following, typical example of each purification step is described in the case of a lot (XW9) and a batch (XB6).

In all of the purification steps, TPO activity was measured using the rat CFU-MK assay system described in <Reference Example>. Unless otherwise noted, all purification

steps were carried out at 4\_C, excluding the reverse phase chromatographies, and Superdex 75pg gel filtration in the presence of surfactant, which were carried out at room temperature. Protein assay was carried out by a Coomassie dye binding assay (a reagent system manufactured by PIERCE, catalog No. 23236X) or a method using bicinchoninic acid (a reagent system manufactured by PIERCE, catalog No. 23225).

An outline of the purification is shown in Table 1.

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ve Total Activity ty activity recovery (%)	2 864600 100	704000	905 3834000 443 847 2339000 271	38 2055000 238
al Relative in activity	8 8		_	1 20000 2 7838
Total protein (mg)	49300	314400	4236	)) 51 )) 262
Step	Total plasma Ca-Treated plasma/G25	Q-Sepharose FF WGA-Agarnse	TSK-gel AF-Blue 650MH Phenyl-Sepharose 6 FF/LS	S-200HR F3 (low mol. TPO) S-200HR F2 (high mol. TPO)
		< Ion exchange >	< Triazine dye > < Hydrophobic >	< Gel filtration>

# Low molecular TPO (from S-200HR F3)

Activity recovery (%)	911	43	35	22	53	
Total activity	1007000	371000	298400	193600	250300	
Relative activity	20000	130000	800000	4890000	149000000	
Total protein (mg)	50.3300	2.8540	0.3730	0.0396	0.0017	
Step	<gel filtration=""> S-200HR F3 (low mol. TPO)</gel>	< Reverse phase > YMC Protein-RP prep	< Reverse phase > YMC CN-AP		Λ	(under non-reduction cond.)

High molecular TPO (from S-200HR F2)

Acti	(%) 233	300 236 50 146 117
Total activity r	2015000	2588000 2041000 434000 1260000 990000
Relative activity	7840	227000 1750000 700000 20000000 330000000
Total protein	(mg) 257.0000	11.4000 1.1660 0.6200 0.0630 0.0030
Stcp	S-200HR F2 (high mol TPO)	
	< Gel filtration >	< Reverse phase >   < Gel filtration >   < Reverse phase >   < Italian phase >   < Electrophoresis >   < Electrophoresis >

## (1) Calcium chloride treatment, centrifugation and protease inhibitor treatment of rat blood plasma

#### in the case of lot No. XW9

XRP (742 ml; protein concentration, 54.8 mg/ml; total protein, 40,686 mg) corresponding to about 100 rats, which had been stored at -80°C, was thawed and dispensed into polypropylene centrifugation tubes (manufactured by Nalgene). Calcium chloride powder was added to each tube to a final concentration of 100 mM. After overnight incubation at 4°C, the resulting mixture was centrifuged at 8,000 rpm for 60 minutes. To the resulting TPO-active supernatant fluid (742 ml; protein concentration, 54.9 mg/ml; total protein, 40,740 mg) was added a protease inhibitor p-APMSF ((p-aminodiphenyl)methanesulfonyl fluoride hydrochloride, manufactured by Wako Pure Chemical Industries, Ltd., catalog No. 010-10393) to a final concentration of 1 mM. The thus obtained sample was used in the following Sephadex G-25 column buffer exchange step.

In this manner, calcium chloride/p-APMSF treatment of the whole XRP derived from about 1,100 rats (total volume, 8,184 ml; total protein, 493,000 mg) was effected by dividing it into 11 lots, each lot being equivalent to about 100 rats, and carrying out treatment of these lots one by one. The resulting 11 samples were processed separately in the subsequent Sephadex G-25 column step.

# (2) <u>Sephadex G-25 <buffer exchange></u> In the case of lot No. XW9

The supernatant fluid obtained after the calcium treatment of the step (1) (742 ml; protein concentration, 54.9 mg/ml; total protein, 40,740 mg) was applied at a flow rate of 40 to 70 ml/min to a Sephadex G-25 column (manufactured by

Pharmacia Biotech, catalog No. 17-0033-03; diameter, 11.3 cm; bed height, 47 cm) which had been equilibrated in advance with 20 mM Tris-HCl, pH 8. Elution was carried out with the same buffer. A 1,300 ml portion of the eluate before the elution of protein was discarded. When UV absorption was detected in the eluates, fractions were pooled until electric conductivity reached 500 µS/cm, thereby recovering a TPOactive protein fraction exchanged by 20 mM Tris-HCl, pH 8 (1,377 ml; protein concentration, 27.56 mg/ml; total protein, 37,882 mg; protein yield, 93%). Relative activity of TPO in the fraction was found to be 2.3.

As the result of the Sephadex G-25 column treatment of the whole lots of samples, a total of 21,117 ml of the Sephadex G-25 column TPO-active fraction was obtained (total protein, 480,300 mg; average relative activity, 2; total activity, 864,600).

## (3) Q-Sepharose FF <strong anion exchange chromatography> In the case of lot No. XW9

The TPO-active fraction obtained in the above step (2) by the Sepharose G-25 treatment (1,377 ml; protein concentration, 27.5 mg/ml; total protein, 37,841 mg; relative activity, 2.3) was applied to a Q-Sepharose FF (manufactured by Pharmacia Biotech, catalog No. 17-0510-01; diameter, 5 cm; bed height, 27 cm) at a flow rate of 40 ml/min, eluted with 20 mM Tris-HCI (pH 8) and a fraction F1 which passed through the column was pooled (3,949 ml; protein concentration, 0.98 mg/ml; total protein, 3,870 mg; relative activity, 0).

Next, the buffer was changed to 20 mM Tris-HCI (pH 8) containing 175 mM NaCl to elute a TPO-active fraction F2 (4,375 ml; protein concentration, 5.36 mg/ml).

Finally, a fraction F3 (1,221 ml; protein concentration, 3.9 mg/ml; total protein, 4,783 mg; relative WO 95/21919 - 71 - PCT/JP95/00208

activity, 3.8) was eluted with 20 mM Tris-HCI (pH 8) containing 1,000 mM NaCl. Total protein in the TPO-active fraction F2 was found to be 23,440 mg, and protein yield of F2 by this step was 61.9%. In addition, relative activity of TPO was increased to 6.8.

As the result of the application of the whole lots of the Sephadex G-25 TPO-active fractions to the Q-Sepharose FF, a total of 35,842 ml of the Q-Sepharose FF TPO-active fraction F2 was obtained (total protein, 314,384 mg; average relative activity, 9; total activity, 2,704,000).

## (4) Wheat germ agglutinin (WGA)-Agarose <lectin affinity chromatography> In the case of lot No. XW9

The TPO-active fraction F2 obtained in the above step (3) by the Q-Sepharose FF treatment was divided into three portions and applied to a WGA-Agarose (manufactured by Honen Corp., catalog No. 800273; diameter, 5 cm; bed height, 22.5 cm) at a flow rate of 5 ml/min, and eluted with Dulbecco's isotonic phosphate buffer (DPBS). A fraction F1 which passed through the column was pooled (9,336 ml; protein concentration, 2.30 mg/ml; total protein, 21,407 mg; relative activity, 6.9).

Next, the buffer was changed to 20 mM sodium phosphate buffer (pH 7.2) containing 0.2 M N-acetyl-D-glucosamine (GlcNAc, manufactured by Nacalai tesque, catalog No. 005-20), 150 mM NaCl and 0.02% sodium azide, and the resulting eluates were pooled and concentrated using an ultrafiltration unit (Omega Ultrasette, nominal molecular weight cutoff of 8,000; manufactured by Filtron), thereby obtaining a WGA-Agarose adsorbing TPO-active fraction F2 (2,993 ml; protein concentration, 0.376 mg/ml).

Total protein in the TPO-active fraction F2 was found to be 1,125 mg, and protein yield of F2 by this step was

4.8%. In addition, relative activity of TPO was increased to 101. The thus obtained F2 fraction was stored at -80°C.

As the result of the application of the whole lots of the Q-Sepharose FF TPO-active F2 fractions to the WGA-Agarose, a total of 33,094 ml of the WGA-Agarose TPO-active fraction F2 was obtained (total protein, 15,030 mg; average relative activity, 132; total activity, 1,987,000).

# (5) <u>TSK-gel AF-BLUE 650 MH <triazine dye affinity</u> chromatography≥

### In the case of batch No. XB6

The WGA-Agarose adsorbing TPO-active fraction of lot No. XW8 and the WGA-Agarose adsorbing TPO-active fraction F2 of lot No. XW9 obtained in the above step (4) starting from 215 rats-equivalent XRP were combined as a batch No. XB6 (5,947 ml, protein concentration, 0.388 mg/ml; total protein, 2,319 mg; relative activity, 150).

A 5,974 ml portion of the combined sample was mixed with 0.85 moles of NaCl for 1,000 ml (296.76 g in total) to make a 6,132 ml of solution having a final NaCl concentration of 0.822 M, and the resulting solution was applied at a flow rate of 7 ml/min to a TSK-gel AF-BLUE 650 MH column (TOSOH CORP., catalog No. 08705; diameter, 5 cm; bed height, 23 cm) which had been equilibrated in advance with 20 mM sodium phosphate buffer (pH 7.2) containing 1 M NaCl.

After completion of the application, protein was eluted using 20 mM sodium phosphate buffer (pH 7.2) containing 1 M NaCl at a flow rate of 10 ml/min. The resulting eluates were pooled and concentrated using an ultrafiltration unit (Omega Ultrasette, nominal molecular weight cutoff of 8,000), thereby obtaining a passed-through fraction F1 (543 ml; protein concentration, 2.05 mg/ml; total protein, 1,112 mg; relative activity, 31).

Next, the elution buffer was changed to 2 M NaSCN to obtain an eluted TSK-gel AF-BLUE 650 MH adsorbing TPO-active fraction F2 (1,427 ml; protein concentration, 0.447 mg/ml).

Total protein in the TPO-active fraction F2 was found to be 638 mg, and protein yield of F2 by this step was 27.5%. In addition, relative activity of TPO was increased to 1.500.

As the result of the application of all of the batches of the WGA-Agarose adsorbing TPO-active F2 fractions to the TSK-gel AF-BLUE 650 MH, a total of 10,655 ml of the TSK-gel AF-BLUE 650 MH TPO-active fraction F2 was obtained (total protein, 4,236 mg; average relative activity, 905; total activity, 3,834,000).

# (6) Phenyl Sepharose 6 FF/LS <hydrophobic interaction chromatography≥

### in the case of batch No. XB6

A 1,424 ml portion of the TSK-gel AF-BLUE 650 MH TPO-active fraction F2 (1,424 ml; protein concentration, 0.447 mg/ml; total protein, 638 mg; relative activity, 1,500) was mixed with 1.5 moles of ammonium sulfate powder per 1,000 ml (282.2 g in total) to make a 1,581 ml of solution having a final ammonium sulfate concentration of 1.35 M.

The resulting solution was applied at a flow rate of 7 ml/min to a Phenyl Sepharose 6 FF (Low Sub) column (Pharmacia Biotech, catalog No. 17-0965-05; diameter, 5 cm; bed height, 10 cm) which has been equilibrated in advance with 50 mM sodium phosphate buffer (pH 7.2) containing 1.5 M ammonium sulfate. After completion of the application, elution was carried out using 36 mM sodium phosphate buffer containing 0.8 M ammonium sulfate at a flow rate of 10 ml/min The resulting eluates (about 3,160 ml) were pooled and concentrated using an ultrafiltration unit (Omega

Ultrasette, nominal molecular weight cutoff of 8,000), thereby obtaining a fraction F1 (485 ml; protein concentration, 0.194 mg/ml; total protein, 94.2 mg; relative activity, 0).

Next, the elution buffer was changed to 20 mM sodium phosphate buffer (pH 7.2) to obtain an eluted TPO-active fraction F2 (about 3,500 ml). The eluted fraction was concentrated using an ultrafiltration unit (Omega Ultrasette, nominal molecular weight cutoff of 8,000) and a sample for assay was taken out. At this stage, protein concentration and total protein of the TPO-active fraction F2 (220 ml) were found to be 1.45 mg/ml and 319 mg, respectively, and protein yield of F2 by this step was 50.0%. Relative activity of TPO was found to be 1,230.

As the result of the application of all of the batches of the TSK-gel AF-BLUE 650 MH TPO-active F2 fractions to the Phenyl Sepharose 6 FF/LS, a total of 1,966 ml of the Phenyl Sepharose 6 FF/LS TPO-active fraction F2 was obtained (total protein, 2,762 mg; average relative activity, 847; total activity, 2,339,000).

### (7) <u>Sephacryl S-200 HR <gel filtration chromatography></u> In the case of batch No. XB6 (cf. Fig. 1)

The Phenyl Sepharose 6 FF/LS TPO-active fraction F2 (217 ml; protein concentration, 1.45 mg/ml; total protein, 315 mg; relative activity, 1,230) was mixed with 144.8 ml of 5 M NaCl solution to make a 362 ml solution having a final NaCl concentration of 2 M, and the resulting solution was concentrated to about 50 ml using an ultrafiltration unit with YM 3 membrane (76 mm in diameter, manufactured by Amicon Corp.).

To this was added the same volume (50 ml) of 8 M urea, thereby obtaining about 100 ml of a solution containing 1 M NaCl and 4 M urea as final concentrations. This was concentrated to about 80 ml, made into a sample of 88.78 ml

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and then applied to a Sephacryl S-200 HR column (manufactured by Pharmacia Biotech, catalog No. 17-0584-01; 7.5 cm in diameter and 100 cm in bed height).

Thereafter, elution was effected with DPBS at a flow rate of 3 ml/min, and the eluates after a void volume of 1,200 ml were collected in 45 ml portions in 60 polypropylene tubes. Assay was carried out every two tubes and the rest were stored at -85°C until the Sephacryl S-200 HR treatment of all samples derived from 1,100 rats was completed. Based on the assay results, fractions of the batch No. XB6 were grouped as follows.

- (F1) tube numbers 1 to 15 (fractions around void volume; molecular weight, 94,000 or more)
- (F2) tube numbers 16 to 26 (molecular weight, 94,000 to 33,000)
- (F3) tube numbers 27 to 44 (molecular weight, 33,000 to 3,000)
- (F4) tube numbers 45 to 55 (molecular weight, 3,000 or less)

In this manner, all batches of the TPO-active F2 fractions obtained by Phenyl Sepharose 6 FF/LS were separately subjected to Sephacryl S-200 HR, assayed and stored at -85°C. After completion of the S-200 HR treatment of all batches and just before the subsequent operation of reverse phase chromatography (YMC-Pack PROTEIN-RP), these stored samples were thawed and concentrated using an ultrafiltration unit with YM 3 membrane (76 mm in diameter, manufactured by Amicon Corp.) to obtain the following two samples. The concentrated sample of the TPO-active fraction F2 obtained by the Sephacryl S-200 HR is referred to as "high molecular TPO sample F2" hereinafter, and that of F3 as "low molecular TPO sample F3".

The high molecular TPO sample F2 and the low molecular TPO sample F3 are pooled fractions of different elution areas in the gel filtration chromatography as a matter of convenience, and the term "high molecular" and "low molecular" therefore may not mean their true molecular weights.

Molecular weight Total volume Total volume	High molecular TPO sample F2 94,000 - 33,000 3,420 ml 293 ml	Low molecular TPO sample F3 33,000 - 3,000 6,480 ml 280 ml
(after concentration)		
Total protein	262 mg	51.3 mg
Average relative act	ivity 7,838	20,000
Total activity	2,055,000	1,020,000

The low molecular TPO sample F3 and the high molecular TPO sample F2 are separately subjected to the subsequent purification steps.

Purification of the low molecular TPO sample F3 is described in the following steps (8) to (11).

### (8) YMC-Pack PROTEIN-RP < reverse phase chromatography>

The low molecular TPO sample F3 (total protein, 50.3 mg; protein concentration, 0.184 mg/ml; relative activity, 20,000; total activity, 1,007,000; total volume, 274 ml) obtained in the above step (7) was mixed with a solvent A (0.025% trifluoroacetic acid (TFA)) and a solvent B (1-propanol containing 0.025% TFA) to obtain a solution having a total volume of 508.63 ml and final propanol, TFA and protein concentrations of about 20%, 0.012% and 0.0989 mg/ml, respectively. Insoluble materials formed during the preparation of this solution were separated by centrifugation, and the resulting supernatant was divided into two 254.3 ml

(25.2 mg protein) portions and applied at a flow rate of 2 ml/min to a column packed with YMC-Pack PROTEIN-RP (manufactured by YMC, catalog No. A-PRRP-33-03-15; 3 cm in diameter and 7.5 cm in bed height) which has been equilibrated in advance with 30% B. The precipitate resulting from the centrifugation was dissolved in 20 ml of 20 mM sodium acetate (pH 5.5) containing 5 mM CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; manufactured by Dojindo Laboratories, catalog No. 75612-03-3) and also applied to the column.

After the sample loading, about 50 ml of a solvent (solvents A:B = 3:1) was passed through the column to obtain a passed-through fraction. Next, a developing program (120 minutes of linear gradient from 30% B to 45% B) was started, and the eluates were collected in 36 polypropylene tubes in 10 ml portions. This process was repeated again for the remaining sample using the same collection tubes, thus obtaining a total of 36 fraction tubes each containing 20 ml eluate. The passed-through fraction was directly concentrated to 20 ml using an ultrafiltration unit with YM 3 membrane (76 mm in diameter, manufactured by Amicon Corp.).

A 0.1 ml portion of each of the passed-through fraction and the 20 ml fractions of tube numbers 1 to 36 was mixed with 20 µl of 5% BSA, dried by evaporation, dissolved in 0.25 ml of IMDM assay culture medium and then assayed to specify TPO-active fractions. As the result, the TPO activity was found in the fractions of tube numbers 17 to 27 (36.0 to 43.0% propanol concentration range) which were combined and used as a low molecular TPO sample F3-derived YMC-Pack PROTEIN-RP TPO-active fraction F2. This fraction was stored at -85°C until its use in the subsequent YMC-Pack CN-AP step.

Low molecular TPO sample F3-derived YMC-Pack PROTEIN-RP TPO-

active fraction F2

Total volume 220 ml
Protein concentration 0.0130 mg/ml
Total protein 2.85 mg
Relative Activity 130,000
Total activity 371,000

### (9) YMC-Pack CN-AP < reverse phase chromatography>

A 214.9 ml portion of the low molecular TPO sample F3-derived YMC-Pack PROTEIN-RP TPO-active fraction F2 (total protein, 2.79 mg; protein concentration, 0.0130 mg/ml; relative activity, 130,000; total activity, 36,300) obtained in the above step (8) was mixed with 0.6 ml of 50% glycerol and concentrated to 1.8 ml. The concentrate was finally adjusted to a volume of 5 ml containing 20% or less of propanol and about 6% of glycerol.

The concentrate was divided into 5 portions for use in the following column operation (0.555 mg protein and 1 ml volume for each operation). Each of the thus divided samples was applied at a flow rate of 0.6 ml/min to a column packed with YMC-Pack CN-AP (manufactured by YMC, catalog No. AP-513; 6 mm in diameter and 250 mm in bed height) which has been equilibrated in advance with 15% B, using 0.1% TFA as the solvent A and 0.05% TFA-containing 1-propanol as the solvent B. After the application, propanol concentration was increased from 15% B to 25% B, and 65 minutes of linear gradient from 25% B to 50% B was carried out. After completion of the final (5th) operation, 1 ml of a solution having the same composition excluding protein was applied to the column and developed in the same manner to recover TPO activity retained in the column. Since the same polypropylene tubes were used to pool the eluates of the 6 operations, a total of 44 tubes each containing 7.2 ml eluate were obtained.

A 30  $\mu$ l portion of each of the thus obtained fractions (1/240 portion of each fraction) was mixed with 20  $\mu$ l of 5% BSA, dried by evaporation, dissolved in 0.24 ml of IMDM assay culture medium and then assayed to specify TPO-active fractions. As the result, strong TPO activity was found in the fractions of tube numbers 28 to 33 (37.0 to 42.0% propanol concentration range) which were combined and used as a low molecular TPO sample F3-derived YMC-Pack CN-AP main TPO-active fraction FA.

Low molecular TPO sample F3-derived YMC-Pack CN-AP TPO-active

fraction FA

Total volume 43.20 ml
Protein concentration 0.00863 mg/ml
Total protein 0.373 mg
Relative Activity 800,000
Total activity 298,400

# (10) Capcell Pak C1 300 <final reverse phase chromatography>

A 43.12 ml portion of the 43.20 ml low molecular TPO sample F3-derived TPO-active fraction FA (total protein, 0.372 mg; protein concentration, 0.00863 mg/ml; relative activity, 800,000; total activity, 297,500) obtained in the above step (9) was mixed with 0.2 ml of 50% glycerol and concentrated to obtain 0.1 ml of glycerol solution.

This solution was mixed with 2 ml of a solution of solvent A (0.1% TFA): solvent B (1-propanol containing 0.05% TFA) = 85:15 (15% B) to prepare 2.1 ml of sample containing about 14% propanol, about 4.8% glycerol and 0.177 mg/ml of protein. The thus prepared sample was applied to a column packed with Capcell Pak C1 300A (manufactured by Shiseido Co., Ltd., catalog No. C1 TYPE:SG300A; 4.6 mm in diameter and

250 mm in bed height) which has been equilibrated in advance with 15% B and developed by 65 minutes of linear gradient from 27% B to 38% B at a flow rate of 0.4 ml/min. The eluates were collected in 72 polypropylene tubes in 0.6 ml portions.

A 3  $\mu$ l portion of each of the thus obtained fractions (1/200 portion of each fraction) was mixed with 20  $\mu$ l of 5% BSA, the medium was exchanged to 225  $\mu$ l of IMDM assay culture medium and the thus 75 times diluted fraction was assayed.

A 1 μl portion of each fraction (1/600 fraction) was aliquotted for use in electrophoresis, dried by evaporation and treated at 95 °C for 5 minutes with 10 μl of a nonreducing sample buffer for SDS-PAGE. The thus treated sample was subjected to SDS-PAGE using a 15-25% SDS-polyacrylamide precast gel (manufactured by Daiichi Pure Chemicals Co., Ltd.) and then stained with a silver staining kit of 2D-Silver Stain·II "DAIICHI" (manufactured by Daiichi Pure Chemicals Co., Ltd., catalog No. 167997; to be referred to as "silver staining kit" hereinafter). As to molecular weight markers, [DAIICHI]-III Low Molecular Weight Markers (manufactured by Daiichi Pure Chemicals Co., Ltd., catalog No. 181061; to be referred to as "DPCIII" hereinafter) were used.

As the result of the above assay, significant TPO activity was found in the fractions of tube numbers 35 to 43 (30.0 to 32.5% propanol concentration range). Of these, fractions from tube numbers 36 to 42 (30.5 to 32.0% propanol concentration range) were combined and used as the main TPO-active fraction FA. The results are shown in Fig. 2.

When the protein content deduced from the chromatogram was compared with the assay results, the thus obtained fraction was found to have a total protein of 39.6  $\mu$ g, a protein concentration of 9.4  $\mu$ g/ml, a relative activity of 4,890,000 and a total activity of 193,600. Examination of the SDS-PAGE patterns of the TPO-active fraction numbers 36 to

42 revealed the presence of a band whose staining density correlates with the activity strength. In addition, apparent molecular weight of this non-reduced band was found to be 17,000 to 19,000 when compared with those of the standard molecular weight proteins on the same gel, which were reduced, thus revealing that this band is a strong candidate of TPO.

# (11) Extraction of TPO-active protein from electrophoresis gel <15% SDS-PAGE>

### Example of the analysis of TPO-active fraction FA

Of the 4,200  $\mu$ l of the low molecular TPO sample F3-derived TPO-active fraction FA (total protein, 39.6  $\mu$ g; protein concentration, 9.4  $\mu$ g/ml; relative activity, 4,890,000; total activity, 193,600) obtained in the above step (10), 5.5  $\mu$ l (1/764 fraction) for use in active protein extraction and 2.5  $\mu$ l (1/1680 fraction) for use in silver staining were transferred into respective sample tubes, dried by evaporation, reacted with 10  $\mu$ l of a nonreducing sample buffer for SDS-PAGE at 37  $\cdot$ c for 1 hour and then allowed to stand still for 18 hours at room temperature, thereby effecting the SDS reaction.

Pre-stained Low Range Marker (manufactured by Bio-Rad Laboratories, Inc., 161-0305) and the aforementioned DPCIII were used as molecular weight markers. Making use of a microslab gel, 15% SDS-PAGE of these samples was carried out at 4°C in accordance with a usually used procedure (Laemmli, Nature, vol.227, pp.680 - 685, 1970). After completion of the electrophoresis, gel portions to be subjected to silver staining were immediately cut out with a knife, put into a fixing solution and then stained using the aforementioned silver staining kit.

Separately from this, all molecular weight range of the gel to be used for the detection of the activity was cut

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with a knife into 34 slices, and each gel slice having a width of 1.5 to 2.5 mm was smashed by a slightly modified method of Kobayashi (Kobayashi, M., Seikagaku (Biochemistry), vol.59, no.9, 1987, published by the Japanese Biochemical Society). Each gel slice thus smashed into minute fragments was mixed with 0.3 ml of an extraction buffer (20 mM Tris-HCl, pH 8, 500 mM NaCl, 0.05% BSA) and shaken for 6 hours at 4 c to effect extraction.

To this was added 500 mM potassium phosphate (pH 6.8) to a final concentration of 20 mM. After 1 hour of shaking at 4 °C, the resulting mixture was transferred into an Ultra Free C3GV 0.22  $\mu m$  filtration unit (manufactured by Millipore Corp., UFC3 OGV 0S) and centrifuged at 1,000 x g (4,000 rpm) for 15 minutes to remove precipitated SDS and recover the resulting supernatant fluid. The filtrate was transferred into an Ultra Free C3-LGC ultrafiltration unit (nominal molecular weight cutoff of 10,000, manufactured by Millipore Corp., UFC3 LGC 00) and centrifuged at 3,000 x g (7,000 rpm). When volume of the concentrated solution reached about 50  $\mu$ l, 300  $\mu$ l of 20 mM sodium phosphate buffer (pH 7.2) was added and again subjected to the ultrafiltration.

The ultrafiltration using 300  $\mu$ l of 20 mM sodium phosphate buffer was repeated twice to remove remaining SDS. Thereafter, a similar step was repeated to exchange to the assay culture medium for preparation of a sample (300  $\mu$ l) which was subsequently sterilized and subjected to TPO activity measurement.

Three protein bands were detected clearly by the silver staining which showed apparent molecular weights of about 17,000 to 19,000, 14,000 and 11,000 based on the DPCIII molecular weight markers.

In the previous step (10), a band having a correlation between activity strength and staining density and having an apparent molecular weight of about 17,000 to

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19,000 was observed in the electrophoresis of the Capcell Pak C1 column TPO-active fraction. In the experiment of this step (11), TPO activity was found also in the band having an apparent molecular weight of about 17,000 to 19,000 (cf. Fig. 3).

On the basis of the above results, it was confirmed that the TPO-active protein was purified in the active fraction of Capcell Pak C1 300A column to a detectable level on the electrophoresis gel. Based on the silver-stained density of the band obtained by 15% SDS-PAGE, the amount of the candidate TPO protein (an apparent molecular weight of about 17,000 to 19,000) in the total TPO-active fraction was determined to be about 1.7  $\mu g$ .

Purification of the high molecular TPO sample F2 is described in the following steps (12) to (15).

### (12) YMC-Pack PROTEIN-RP < reverse phase chromatography>

The high molecular TPO sample F2 (total protein, 257 mg; protein concentration, 0.894 mg/ml; relative activity, 7,840; total activity, 2,015,000; total volume, 287 ml) obtained in the above step (7) was mixed with 95.8 ml (1/3 volume of the sample) of a solvent B (1-propanol containing 0.025% TFA) to obtain a solution having a total volume of 383 ml and final propanol. TFA and protein concentrations of about 25%, 0.006% and 0.671 mg/ml, respectively. A solution of 0.025% TFA was used for a solvent A. Insoluble materials formed during the preparation of input sample were separated by centrifugation, and the resulting supernatant fluid was divided into six 62.3 ml (42.8 mg protein) portions and applied at a flow rate of 2 ml/min to a column packed with YMC-Pack PROTEIN-RP (manufactured by YMC, catalog No. A-PRRP-33-03-15; 3 cm in diameter and 7.5 cm in bed height) which has been equilibrated in advance with 30% B. The precipitate resulting from the centrifugation was dissolved in 10 ml of 20 mM WO 95/21919

sodium acetate (pH 5.5) containing 5 mM CHAPS and also applied to the column.

After the sample loading, about 50 ml of a solvent (solvents A:B = 3:1) was passed through the column to obtain a passed-through fraction. Next, a developing program (120 minutes of linear gradient from 30% B to 45% B) was started, and the eluates were collected in 24 polypropylene tubes in 15 ml portions. This process was repeated for the 6 divided samples using the same collection tubes, thus obtaining a total of 24 fraction tubes each containing 90 ml eluate. The passed-through fraction and the tube number 1 fraction were combined and directly concentrated to 90 ml using an ultrafiltration unit with YM3 membrane (76 mm in diameter, manufactured by Amicon Corp.).

A 0.3 ml portion of each of the passed-through fraction plus tube number 1 fraction and the fractions of tube numbers 2 to 24 was mixed with 10 µl of 5% BSA, dried by evaporation, dissolved in 0.30 ml of IMDM assay culture medium and then assayed to specify TPO-active fractions. As the result, the TPO activity was found in the fractions of tube numbers 10 to 15 (34.0 to 39.5% propanol concentration range) which were combined and used as a high molecular TPO sample F2-derived YMC-Pack PROTEIN-RP TPO-active fraction F2. This fraction was stored at -85°C until its use in the subsequent YMC-Pack CN-AP step.

High molecular TPO sample F2-derived YMC-Pack PROTEIN-RP TPO-

#### active fraction F2

Total volume	540 ml
Protein concentration	0.021 mg/ml
Total protein	11.4 mg
Relative Activity	227,000
Total activity	2,588,000

## (13) Superdex 75 pg <gel filtration chromatography in the presence of CHAPS>

A 538.2 ml portion of the high molecular TPO sample F2-derived YMC-Pack PROTEIN-RP TPO-active fraction F2 (total protein, 11.3 mg; protein concentration, 0.021 mg/ml; relative activity, 227,000; total activity, 2,565,000) obtained in the above step (12) was mixed with 0.6 ml of 50% glycerol and concentrated by evaporation. To this was added 18 ml of 20 mM CHAPS, followed by stirring and a subsequent 41 hour incubation at 4 c. Thereafter, the first sample was applied to a column packed with HiLoad 26/60 Superdex 75 pg (manufactured by Pharmacia Biotech, catalog No. 17-1070-01; 2.6 cm in diameter and 60 cm in bed height) and eluted with DPBS containing 5 mM CHAPS at a flow rate of 1 ml/min. A 4 ml portion of each sample (protein concentration, 0.466 mg/ml; protein, 1.86 mg) was applied to the column.

In this case, the Superdex 75 column operation was repeated 6 times by dividing the entire YMC-Pack PROTEIN-RP TPO-active fraction into 6 portions. The eluates of each operation were collected in 5 ml portions in 45 tubes, thus finally obtaining 45 fractions each containing 30 ml eluate after completion of the 6 column operations.

A 0.1 ml portion of each of the thus obtained fractions was mixed with 10  $\mu$ l of 5% BSA, dried by evaporation, dissolved in 0.25 ml of IMDM assay culture medium and then assayed to specify TPO-active fractions. As the result, the TPO activity was found in the fractions of tube numbers 13 to 31 (molecular weight range of from 78,000 to 3,000) which were combined and used as a high molecular TPO sample F2-derived Superdex 75 pg TPO-active fraction F2.

High molecular TPO sample F2-derived Superdex 75 pg TPO-active

fraction F2

Total volume 540 ml
Protein concentration 0.00216 mg/ml
Total protein 1.17 mg
Relative Activity 1,750,000
Total activity 2,041,000

### (14) YMC-Pack CN-AP <reverse phase chromatography>

A 513.2 ml portion of the high molecular TPO sample F2-derived Superdex 75 pg TPO-active fraction F2 540 ml (molecular weight, 78,000 - 3,000) (total protein, 1.11 mg; protein concentration, 0.00216 mg/ml; relative activity, 1,750,000; total activity, 1,943,000) obtained in the above step (13) was mixed with 1/10 volume of a solvent B (1-propanol containing 0.05% TFA) and applied at a flow rate of 0.6 ml/min to a column packed with YMC-Pack CN-AP (manufactured by YMC, catalog No. AP-513; 6 mm in diameter and 250 mm in bed height) which has been equilibrated in advance with 15% B, using a solvent A (0.1% TFA) and the solvent B. After the application, propanol concentration was increased from 15% B to 25% B, and 65 minutes of linear gradient from 25% B to 50% B was carried out.

Prior to the commencement of the YMC-Pack CN-AP column chromatography, a 1/20 portion of the total input sample was subjected to a trial operation in order to confirm proper recovery of the activity. Thereafter, the remaining 19/20 portion was divided into 2 samples to carry out the operation twice. In other words, the column operation was repeated three times. Since the same 44 polypropylene tubes were used to pool the eluates of the 3 operations, a total of 44 tubes each containing 3.6 ml eluate were obtained.

A 5  $\mu$ l portion of each of the thus obtained fractions (1/720 portion of each fraction) was mixed with 0.25 ml of IMDM assay culture medium and then assayed to specify TPO-active fractions. As the result, markedly strong TPO activity was found in the fractions of tube numbers 24 to 30 (36.0 to 42.0% propanol concentration range) which were combined and used as a high molecular TPO sample F2-derived YMC-Pack CN-AP main TPO-active fraction FA.

High molecular TPO sample F2-derived YMC-Pack CN-AP TPO-active

### fraction FA

Total volume	25.20 ml
Protein concentration	0.0246 mg/ml
Total protein	0.620 mg
Relative Activity	700,000
Total activity	434,000

# (15) Capcell Pak C1 300A <final reverse phase chromatography>

A 24.66 ml portion of the 25.20 ml high molecular TPO sample F2-derived YMC-Pack CN-AP TPO-active fraction FA (total protein, 0.606 mg; protein concentration, 0.0246 mg/ml; relative activity, 700,000; total activity, 424,000) obtained in the above step (14) was mixed with 0.4 ml of 50% glycerol and concentrated by evaporation.

In this way, 2 ml of a concentrated sample was obtained with a propanol concentration of a few percent, a glycerol concentration of 10% and a protein concentration of 0.303 mg/ml. This was applied to a column packed with Capcell Pak C1 300A (manufactured by Shiseido Co., Ltd., catalog No. C1 TYPE:SG300A; 4.6 mm in diameter and 250 mm in bed height) which has been equilibrated in advance with 15% B, using a solvent A (0.1% TFA) and a solvent B (1-propanol

containing 0.05% TFA), and developed by 65 minutes of linear gradient from 27% B to 38% B at a flow rate of 0.4 ml/min. The eluates were collected in 72 polypropylene tubes in 0.6  $\mu$ l portions.

A 0.75  $\mu$ l portion of each of the thus obtained fractions (1/800 portion of each fraction) was mixed with 20  $\mu$ l of 5% BSA, the medium was exchanged to 225  $\mu$ l of IMDM assay culture medium and the thus 300 times diluted fraction was assayed.

A 2  $\mu$ l portion of each fraction (1/300 fraction) was sampled for use in electrophoresis, dried by evaporation and treated at 95°C for 5 minutes with 10  $\mu$ l of a nonreducing sample buffer for SDS-PAGE. The thus treated sample was subjected to SDS-PAGE using a 15-25% SDS-polyacrylamide precast gel (manufactured by Daiichi Pure Chemicals Co., Ltd.) and then stained using the aforementioned silver staining kit. DPCIII described in the foregoing was used as molecular weight markers.

As the result of the above assay, significant TPO activity was found in the fractions of tube numbers 33 to 39 (29.5 to 31.5% propanol concentration range). Of these, fractions of tube numbers 34 to 39 (30.0 to 31.5% propanol concentration range) were combined and used as the main TPO-active fraction FA. Examination of the SDS-PAGE pattern of the main TPO-active fraction revealed the presence of a band whose staining density correlates with the activity strength, within an apparent molecular weight range of 17,000 to 19,000 under nonreducing condition, which was similar to the case of the low molecular TPO sample F3-derived TPO-active fraction described in the aforementioned step (10).

<Example 2>
Analysis of partial amino acid sequences of purified rat TPO

Amino acid sequence analysis of the rat TPO candidate protein in the Capcell Pak C1 300A column TPOactive fraction FA obtained in the purification step (10) of Example 1 was carried out in accordance with the procedure of Iwamatsu (Iwamatsu et al., "Shin Kiso Seikagaku Jikken-ho (New Basic Biochemical Experiments)", vol.4, pp.33 - 84, pub. Maruzen; Iwamatsu, A, Seikagaku (Biochemistry), vol.63, no.2, pp.139 - 143, 1991; Iwamatsu, A., Electrophoresis, vol.13, pp.142 - 147, 1992). That is, the sample was subjected to SDS-PAGE and transferred electrically onto a polyvinylidene difluoride (PVDF) membrane. After reduction and S-alkylation of the protein on the PVDF membrane, the thus treated protein was digested into peptide fragments by systematic and stepwise restrictive enzymatic hydrolysis in situ with three proteases, and the resulting peptide fragments on each digestion step were separated and purified by reverse phase chromatography and analyzed for their amino acid sequences by a high sensitivity amino acid sequence determination method. The following describes this process in detail.

Example of the analysis of the TPO candidate protein in the low molecular TPO sample F3-derived Capcell Pak C1 300A TPO fraction FA

(1) Concentration of Capcell Pak C1 300A column TPO fraction FA (tube numbers 36 to 42)

Of the 4,200  $\mu$ l of the low molecular TPO sample F3-derived Capcell Pak C1 300A column TPO-active fraction FA (tube numbers 36 to 42) obtained in the purification step (10) of Example 1 (total protein, 39.6  $\mu$ g; protein concentration, 9.4  $\mu$ g/ml; relative activity, 4,890,000; total activity, 193,600), 4,151  $\mu$ l (98.8% of the total fraction) was used in the amino acid sequence analysis. The total protein deduced from the chromatogram was 39.1  $\mu$ g, of which about

1.6  $\mu g$  was the TPO candidate protein stained with silver after SDS-PAGE and having an apparent molecular weight of about 17.000 to 19.000.

This sample was mixed with glycerol and concentrated by evaporation to obtain 5  $\mu$ l of a glycerol solution. To this were added a non-reducing buffer for SDS-PAGE and 1 M Tris-HCl (pH 8) in order to adjust its pH, thereby obtaining about 25  $\mu$ l of sample containing 200 mM Tris-HCl (pH 8.0), 50 mM Tris-HCl (pH 6.8), 1.1% SDS, 2 mM EDTA, 0.02% bromophenol blue and 30% glycerol.

The thus prepared sample was maintained at room temperature for 14 hours and then treated at 60°C for 5 minutes, in order to effect proper SDS reaction without excess heating.

### (2) Electrophoresis

Micro-slab gels (4.0% acrylamide stacking gel and 15% acrylamide separation gel) were prepared and SDS-PAGE was carried out at room temperature for 2 hours at a constant current of 12.5 mA and then at 17.5 mA. Pre-stained Low Range Marker (Bio-Rad, 161-0305) and DOCIII were used as molecular weight markers. Immediately after the electrophoresis, the resulting protein molecules were transferred on a PVDF membrane (see the following step).

A portion of the sample was also used in another electrophoresis using 15-25% polyacrylamide precast gel (Multi Gel 15/25 manufactured by Daiichi Pure Chemicals Co., Ltd., catalog No. 211072) under non-reducing conditions or after reducing the sample with dithiothreitol (DTT). When the resulting gel was stained using the silver staining kit described in the foregoing, it was confirmed that molecular weight of the protein band expected to be TPO was about 19,000 under a reducing condition and purity of the TPO candidate protein of Capcell Pak C1 300A column was a few

percent. In addition, since mobility of the band varied under non-reducing and reducing conditions, it was suggested that the candidate protein contains at least one disulfide bond.

# (3) Transfer of protein on PVDF membrane by electroblotting and detection of band

Protein transfer on a PVDF membrane (ProBlott, manufactured by Applied Biosystems; catalog No. 400994) was carried out for 1 hour at a constant current of 160 mA (11 to 17 V) using a semi-dry transfer apparatus (Model KS-8460, manufactured by Marysol). A solution consisting of 0.3 M Tris and 20% methanol (pH 10.4) was used as the anolyte, a solution consisting of 25 mM Tris and 20% methanol (pH 10.4) as the transfer membrane solution and a solution consisting of 25 mM Tris, 40 mM aminocaproic acid and 20% methanol (pH 10.4) as the cathlyte.

When the thus transferred membrane was stained with a Ponceau S staining solution (0.1 g of Ponceau S and 1 ml of acetic acid in 100 ml water), a plurality of bands were detected and one of these bands was confirmed to be that of the candidate protein having a molecular weight of about 19,000. This band was cut out for use in the subsequent peptide fragment formation steps.

# (4) Peptide fragment formation, peptide mapping and amino acid sequence analysis

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In order to perform systematic fragmentation of the TPO candidate protein which has been transferred and Salkylated after reduction on the PVDF membrane, stepwise restrictive enzymatic hydrolysis was carried out using the following three proteases. First digestion: Lysyl endopeptidase (*Achromobacter lyticus* m497-1, manufactured by Wako Pure Chemical Industries, Ltd., catalog No. 129-02541)

Second digestion: Endoproteinase Asp-N (manufactured by Boehringer-Mannheim Corp., catalog No. 1054 589)

Third digestion: Trypsin-TPCK (manufactured by Worthington Biochemical, catalog No. 3740)

Peptide fragments obtained by each enzyme digestion were recovered, applied to a Wakosil-II 5C18 C18 reverse phase column (manufactured by Wako Pure Chemical Industries, Ltd., 2.0 mm in diameter and 150 mm in length) and eluted using a solvent A (0.05% TFA) and a solvent B (isopropanol:acetonitrile = 7:3, 0.02% TFA), by 30 minutes of linear gradient of from 1% B to 50% B at a flow rate of 0.25 ml/min and at a column temperature of 30°c. In this way, peptide mappings were made (see Fig. 4). The resulting peptide fragments were recovered and subjected to Edman degradation using a gas phase amino acid sequencer (PPSQ-2, manufactured by Shimadzu Corp.). Thereafter, each amino acid recovered in turn from the sequencer, whose N-terminal has been coupled with PTH, was identified using the C18 reverse phase column chromatography by isocratic elution. The results are summarized in the following.

Amino acid sequences of peptide fragments obtained by the first digestion with lysyl endopeptidase

Fragment	Amino acid sequence
AP3	(K)XYYESZ (X is A, S, G, M or Q and Z is E or K) (SEQ ID NO: 184)
AP6	(K)XRAAZ (X is E or Q and Z is E or N) (SEQ ID NO: 185)
AP7	(K)AGXCSG (cannot be totally identified) (SEQ ID NO: 186)
AP8	(K)XPVPPACDPRLL (X is I, T OR S) (SEQ ID NO: 187)

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.AP12	(K)DSFLA	NDV	/K (SEQ ID NO: 188)
AP5	(cannot	bе	identified)
AP20	(cannot	be	identified)
AP21	(cannot	be	identified)
AP23	(cannot	be	identified)

Amino acid sequences of peptide fragments obtained by the second digestion with endoproteinase Asp-N

Fragment_	Amino acid sequence	·
ASP1	(cannot be identified)	•
ASP2	(cannot be identified)	
ASP6	(cannot be identified)	
ASP11	(cannot be identified)	

Amino acid sequences of peptide fragments obtained by the third digestion with trypsin-TPCK

	The state of the s
Fragment	Amino acid sequence
TP2	(K or R)TLPTXAVP (SEQ ID NO: 189)
TP3	(K or R)TLPTXAVP

In the above sequences, those shown in parentheses in N-termini are amino acid residues which can be deduced from the systematic enzyme digestion.

# (5) Analysis of the homology of obtained amino acid sequences <homology search>

In order to know if the thus obtained amino acid sequences are contained in a known reported protein or there is a protein having similar sequences, they were analyzed using sequence analyzing software Mac Vector (Kodak International Biotechnologies, Inc.). As for the information on known proteins or known genes, Entrez Release 6 data base (National Center for Biotechnology Information, National

Library of Medicine, National Institute of Health, USA, published on August 15, 1993) was used. Data bases included therein are as follows.

Entrez Release 6 database
NCBI-GenBank, August 15, 1993 (Release 78.0)
EMBL, July 15, 1993 (Release 35.0 plus updates)
DDBJ, July 15, 1993
SWISS-PROT, April, 1993 (Release 25.0)
PIR, June 30, 1993 (Release 37.0)
PDB, April, 1993
PRF, May, 1993
dbEST, July 15, 1993 (Release 1.10)
U.S. and European Patents

As the result, the sequence (K)DSFLADVK of AP12 completely coincided with an internal sequence KDSFLADVK of a rat corticosteroid-binding globulin (CBG) precursor [PIR data base accession No. A40066; Smith and Hammond; "Rat corticosteroid-binding globulin: primary structure and messenger ribonucleic acid levels in the liver under different physiological conditions.", *Mol. Endocrinol.*, (1989), 3, 420 - 426].

Further detailed examination revealed that a sequence KQYYESE (SEQ ID NO: 193) having high similarity to the sequence (K)XYYESZ (X is A, S, G, M or Q and Z is E or K) of AP3 is contained in the rat CBG amino acid sequence. In the rat CBG, sequences which correspond to those of AP12 and AP3 are linked to each other thereby forming an internal amino acid sequence KDSFLADVKQYYESE (SEQ ID NO: 190).

With regard to the amino acid sequences of other fragments than AP12 and AP3, no protein or gene was found whose similarity could be taken into consideration.

<Example 3>

Analysis of biological characteristics of TPO derived from blood plasma of thrombocytopenic rats

### (1) In the rat CFU-MK assay system (liquid culture system)

As a typical example, Fig. 5 shows a dose response curve in the case of the use of a TPO sample partially purified from thrombocytopenic rat plasma (TPO-active fraction F2 from YMC Pack Protein-RP column described in the purification step (8) of Example 1-2). When the cultured cells were observed periodically under a microscope, generation and maturation of megakaryocytes, namely increase in the cell size, was recognized, probably together with increase in the As an especially significant number of megakaryocytes. change, process formation by megakaryocytes were found on the 4th day which was the final day of the culturing (they were hardly recognizable on the 3rd day). Such a process formation is called cytoplasmic process formation (Leven and Yee, Blood, vol.69, pp.1046 - 1052, 1987) or proplatelet process formation (Topp et al., Blood, vol.76, pp.912 - 924, 1990), which is considered to be a platelet precursor structure further differentiated from mature megakaryocytes and is considered to be at the final stage of megakaryocyte differentiation so far observable in vitro. Since such a morphological change was observed with a high frequency by using the TPO sample alone, it is possible that this factor alone can stimulate proliferation and differentiation of CFU-MK, can produce mature megakaryocytes and can finally release platelets.

### (2) In the colony assay system

When a TPO sample partially purified from blood plasma of thrombocytopenic rats was examined by the colony assay system using unseparated rat bone marrow cells, cells of each of the separation/concentration steps or a Gpllb/Illa+CFU-MK fraction, the rat plasma-derived TPO stimulated formation of megakaryocyte colonies. In comparison with megakaryocyte colonies induced by other cytokine such as rat

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IL-3, mouse GM-CSF or human EPO, the TPO-induced megakaryocyte colonies are characterized in that each colony consists of smaller numbers of megakaryocytes, but each megakaryocyte is larger in size, which means advanced maturity. In addition, since TPO produced no or few colonies of the other cell lineages, the Meg-CSF activity of TPO can be regarded as megakaryocyte-specific. On the basis of these facts, it is evident that TPO has different biological properties from those of other cytokines such as rat IL-3, mouse GM-CSF and human EPO and exerts unique Meg-CSF activity.

The TPO sample partially purified from blood plasma of thrombocytopenic rats also exerted its Meg-CSF activity on CD34<sup>+</sup>,DR<sup>+</sup> cell fractions derived from human bone marrow cells or human cord blood cells and induced significant numbers of human megakaryocyte colonies, indicating that this factor has no species specificity.

#### <Example 4>

#### Specialization of rat TPO producing cells

#### (1) Screening of rat TPO producing organs

Screening and specialization of rat TPO-producing organs were carried out in order to ensure a mRNA source for use in the cloning of a rat TPO gene. Firstly, bone marrow, lungs, livers and spleens were taken periodically from rats rendered thrombocytopenic by P55 antibody administration, their cells (organ sections in the case of lungs and livers) were cultured and activities in the resulting culture supernatant were examined by the rat CFU-MK assay system. This initial attempt, however, did not yield distinctive results. In consequence, further attempt was made to culture hepatocytes prepared by collagenase perfusion from livers of thrombocytopenic rats induced by P55 antibody administration, taking into consideration a report which indicated a

relationship between the liver and TPO production in rats (Siemensma et al., J. Lab. Clin. Med., vol.86, pp.817 - 833, 1975). When the resulting culture supernatant was applied to a WGA-Agarose column and then the adsorbed fraction was fractionated on a Vydac phenyl reverse phase column, exceedingly similar activity was found at the same position of the rat plasma-derived TPO activity in the rat CFU-MK assay system. Weak but the same activity was also found in culture supernatant of normal rat hepatocytes. These results strongly suggested that the liver would be one of the TPO-producing organs.

### (2) Screening of rat TPO-producing cell lines

On the basis of the above results, screening of rat TPO-producing cell lines was carried out. Firstly, each of 20 rat liver-derived cell lines was cultured in respective subculture liquid medium until the cells reached almost confluent stage, the culture medium was exchanged with the same respective medium supplemented with 5% FCS, and the culturing was continued for 3 days. When each of the resulting culture supernatant was partially purified in accordance with the procedure described in the above step (1) to examine existence of TPO production, the TPO activity was distinctively found in three rat hepatic parenchymal cellderived cell lines, namely McA-RH8994 cells (ATCC deposit No. CRL1602; Becker et al., "Oncodevelopmental Gene Expression", ed. by Fishman and Sell, Academic Press, NY, pp.259 - 270, 1976; purchased from Dainippon Pharmaceutical Co., Ltd.), H4-II-E cells (ATCC deposit No. CRL1548; Pitot et al., Nat. Cancer Inst. Monogr., vol.13, pp.229 - 245, 1964; purchased from Dainippon Pharmaceutical Co., Ltd.) and HTC cells (Thompson et al., Proc. Natl. Acad. Sci. USA, vol.56, pp.296 - 303, 1966; purchased from Dainippon Pharmaceutical Co., Ltd.).

(3) Detailed analysis of TPO activities in McA-RH8994 cells. H4-II-E cells and HTC cells

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Biochemical and biological properties of the TPO-active proteins secreted from these 3 rat cell lines were examined in detail and compared with those of the rat plasmaderived TPO.

McA-RH8994 cells were suspended in alpha-MEM(-) liquid culture medium containing 10% FCS and transferred into a 175 cm<sup>2</sup> plastic tissue culture flask at 1 x 10<sup>6</sup> cells/flask. After 3 days of culturing at 37°c in a 5% CO2 incubator, the medium was exchanged with IMDM culture medium containing 5% FCS and the culturing was continued for additional 3 days to recover the resulting culture supernatant. H4-II-E cells were suspended in Dulbecco's modified Eagle liquid culture medium (glucose 4.5 g/l, to be referred to as "DMEM" hereinafter) containing 10% FCS and transferred into a 175 cm<sup>2</sup> plastic tissue culture flask at 5 x 10<sup>5</sup> cells/flask. After 3 days of culturing at 37°c in a 5% CO2 incubator, the medium was exchanged with IMDM culture medium containing 5% FCS and the culturing was continued for additional 3 days to recover the resulting culture supernatant. Also, HTC cells were suspended in DMEM liquid culture medium containing 5% FCS and transferred into a 175 cm<sup>2</sup> plastic tissue culture flask at  $2.5 \times 10^5$  cells/flask. After 3 days of culturing at 37 c in a 5% CO2 incubator, the medium was exchanged with IMDM culture medium containing 5% FCS and the culturing was continued for additional 3 days to recover the resulting culture supernatant.

Using a 2 liter portion of the thus obtained culture supernatant from each of the 3 cell lines, partial purification of the cell line-derived TPO was carried out in accordance with the procedure for the purification of TPO from XRP described in Example 1-2. The following describes an outline of the results.

Firstly, each culture supernatant was concentrated about 6 times using an ultrafiltration apparatus and the medium was exchanged with 20 mM Tris-HCl buffer (pH 8.0) using a Sephadex G-25 column. The resulting eluate was applied to a Q-Sepharose FF column, the column was washed with 20 mM Tris-HCI (pH 8.0) and then the adsorbed fraction was eluted with 20 mM Tris-HCl (pH 8.0) containing 175 mM NaCl. The thus eluted fraction was applied to a WGA-Agarose column, the column was washed with PBS and the adsorbed fraction was eluted with 20 mM sodium phosphate (pH 7.2) containing 0.2 M GlcNAc and 0.15 M NaCl. The resulting eluate was applied to an TSK-gel AF-BLUE 650MH column, the column was washed with 20 mM sodium phosphate (pH 7.2) containing 1 M NaCl and then the adsorbed fraction was eluted with 2 M The resulting eluate was applied to a Phenyl-Sepharose 6 FF/LS column, the column was washed with 50 mM sodium phosphate (pH 7.2) containing 1.5 M ammonium sulfate followed by 0.8 M ammonium sulfate containing 36 mM sodium phosphate, and then the adsorbed fraction was eluted with 20 mM sodium phosphate (pH 7.2). The thus obtained adsorption fraction was concentrated and then fractionated using a reverse phase Vydac Protein C4 column (manufactured by The Separations Group, catalog No. 214TP51015; 1 cm in diameter and 15 cm in bed height). That is, the sample was applied to the C4 column which has been equilibrated in advance with 20% B and then elution was effected by 90 minutes of linear gradient of from 20% B to 40% B at a flow rate of 1 ml/min, using 0.1% TFA in the developing solvent A and 1-propanol containing 0.05% TFA in the developing solvent B. result, each of the TPO-active proteins derived from these cell lines was eluted at a 1-propanol concentration of 30 to 43%.

When TPO activities in samples of each purification step were measured by the rat CFU-MK assay system, the results showed that TPO activities of the 3 cell

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lines behaved with the similar patterns to those of the XRPderived TPO (cf. Example 1-2). Relative activity, activity yield and the like at each purification step measured by the rat CFU-MK assay system are shown in Table 2. In addition, when TPO activities in the eluates from the final reverse phase column were measured by the rat CFU-MK assay system, TPO activities of the 3 cell lines and the XRP-derived TPO were found in the same peak area. When colony assay of TPO-active fractions from the reverse phase column was carried out using nonadherent cells obtained in the adherence depletion step for the separation and concentration of rat GpIIb/IIIa+ CFU-MK, elution patterns of Meg-CSF activity closely resembled those of TPO activity measured by the rat CFU-MK assay system (see Table 3). In addition, similar to the case of the XRP-derived TPO, each of TPO activities of the 3 cell lines formed no or few colonies of the other cell lineages.

Each of the pooled active fractions from the reverse phase column was subjected to SDS-PAGE in accordance with the procedure described in Example 1-2. When protein was extracted from the resulting gel and TPO activity was measured by the rat CFU-MK assay system, apparent molecular weights of the XRP-derived TPO, McA-RH8994 cellderived TPO and H4-II-E cell-derived TPO were found to be 17,000 to 22,000, 33,000 to 39,000 and 31,000 to 38,000, respectively, and the HTC cell-derived TPO showed apparent molecular weights of 17,000 to 22,000 and 28,000 to 35,000.

Thus, these results revealed that the TPO proteins produced by McA-RH8994 cells, H4-II-E cells and HTC cells are equivalent to the XRP-derived TPO in terms of their biological properties, though their biochemical properties are slightly different from one another in terms of apparent molecular A remarkable finding in the present invention is a weight. possibility that a TPO-active protein molecule contained in blood may be a product of partial digestion at its specific or irregular site in its production cells or after its secretion from the production cells. It also suggests possible existence of TPO genes (mRNA and cDNA) having various lengths.

Table 2 Purification of TPO from rat cell lines

Cell Line	McA-	RH8994			HTC		H4-II-	п	
Step	TP*I	TP* RA*2	TA*3	TP	RA	TA	TP RA	RA	۲ <sub></sub>
•	(mg)			(mg)			(gm)		
Culture supernatant	4806	∞	38450	2760	2	28800	4087	S	20440
Sephadex G25	4824	91	77180	6458	13	77500	4011	2	20050
O-Sepharose FF	1973	70	39460	3112	15	46680	1821	15	27320
WGA-Agarose	76.0	1350	102600	132.0	250	33000	0.08	90	7200
TSK-gel AF-Blue	5.0	12500	62500	9.2	7500	00069	5.4	150	810
HM059									
Phenyl-Sepharose	14.7	200	7350	13.1	2500	32750	H.4	2	1940
6 FF/LS									
Vydac Protein C4	0.23	1	1	0.75	i	1	- - -	1	

\*1, total protein; \*2, relative activity; \*3, total activity

Table 3 Rat megakaryocyte colony formation by rat TPO (reverse phaseC4 column fraction)

Elution	XRP	McA-RH8994	HTC	H4-II-B
propanol	colonics	colonies	colonies	colonies
30.0 - 32.6%	0	33	4	<u>«</u>
32.6 - 34.7%	0	132	61	20
34.7 - 36.7%	<i>L</i> 9	290	291	230
36.7 - 38.8%	70	214	183	103
38.8 - 40.9%	2	15	5	28
40.9 - 43.0%	0	4	0	4

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#### <Example 5>

#### Construction of expression vector (pEF18S) for cDNA library

A vector into which a cDNA fragment can be integrated easily and which can exert a high expression efficiency of the cloned cDNA was constructed for use in the cloning and expression of TPO cDNA. That is, an expression vector pEF18S was constructed from an expression vector pME18S by replacing the SRα promoter with an elongation factor  $1\alpha$  (EF1 $\alpha$ ) promoter which is known as a high expression promoter (cf. Fig. 6). The promoter of elongation factor  $1\alpha$ was obtained by partially digesting 1 µg of an expression vector pEF-BOS (Mizushima et al., Nucleic Acids Res., vol.18, p.5322, 1990) with restriction enzymes Hindll and EcoRI, subjecting the digest to 2% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a DNA fragment of about 1,200 bp and purifying the DNA fragment using a Prep-A-Gene DNA purification kit (manufactured by Bio-Rad Laboratories, Inc.; a kit for use in the selective purification of DNA which is effected by porous silica matrix-aided DNA adsorption, developed on the basis of the procedure reported by Willis et al. in Bio Techniques, vol.9, pp.92-99, 1990). A 100 ng portion of the thus obtained DNA fragment was ligated with 50 ng of an expression vector pME18S (Liu et al., Proc. Natl. Acad. Sci. USA, vol.90, pp.8957 - 8961, 1993) which has been digested in advance with Hindll and EcoRl. Cells of a commercial host strain, Competent High E. coli DH5 (manufactured by Toyobo Co., Ltd.; a competent E. coli strain prepared by a modified method of Hanahan et al., J. Mol. Biol., vol.166, pp.557 - 580, 1983), were transformed with the thus constructed vector. After culturing the transformed cells, 12 colonies were selected at random and plasmid DNA was purified from each colony. A total of 10 clones containing a plasmid of interest (pEF18S) were obtained by analyzing restriction enzyme digestion patterns of these DNA molecules. Thereafter, a clone selected therefrom was cultured to prepare a large quantity of the plasmid DNA.

Purification of the plasmid DNA was carried out basically in accordance with the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). That is, the clone pEF18S obtained above was cultured overnight at 37°c in 50 ml of an LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) containing 50  $\mu g/ml$  of ampicillin, and the resulting cells collected by centrifugation were suspended in 4 ml of TEGlysozyme solution (25 mM Tris-HCl, pH 8, 10 mM EDTA, 50 mM glucose, 0.5% lysozyme). To this were added 8 ml of 0.2 N NaOH/1% SDS solution and then 6 ml of 3 M potassium/5 M acetate solution to suspend the cells thoroughly. centrifugation of the suspension, the resulting supernatant fluid was treated with phenol/chloroform (1:1), mixed with the same volume of isopropanol and then centrifuged. resulting pellet was dissolved in TE solution (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and treated with RNase and then with phenol/chloroform (1:1), followed by ethanol precipitation. The resulting pellet was again dissolved in TE solution to which were subsequently added NaCl and polyethylene glycol 3,000 to their final concentrations of 0.63 M and 7.5%, respectively. After centrifugation, the pellet was dissolved in TE solution and precipitated with ethanol. In this way, about 300  $\mu g$  of the plasmid DNA was obtained. A 100  $\mu g$  portion of the DNA was completely digested with restriction enzymes EcoRI and NotI and subjected to 0.8% agarose gel (manufactured by FMC BioProducts) electrophoresis, and the thus recovered vector fragments were purified using a Prep-A-Gene DNA purification kit (manufactured by Bio-Rad Laboratories, Inc.) to obtain about 55 µg of plasmid DNA which was used in the subsequent cDNA library construction.

#### <Example 6>

### Purification of mRNA from McA-RH8994 cells

McA-RH8994 cells which showed relatively high activity in the colony assay of Example 4 were selected as the material for use in the rat TPO cDNA cloning and subjected to the following experiments.

Isolation of total RNA was carried out basically in accordance with the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). That is, McA-RH8994 cells were grown to confluence in 15 culture dishes of 90 mm in diameter. After removing the liquid medium, cells in each dish were thoroughly suspended in 0.8 ml of a 5 M guanidine solution (5 M guanidine thiocyanate, 5 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, 0.5% sodium sarcosylsulfate), and the resulting suspensions in all dishes were collected in a single tube and the total volume was adjusted to 20 ml with the guanidine solution. resulting mixture, which became viscous due to cell disruption, was subjected to about 20 repetitions of suction/discharge with a 20 ml syringe equipped with a 18G needle and then 21G needle until the mixture became almost non-viscous. A 18 ml portion of 5.7 M CsCl-0.1 M EDTA (pH 7.5) was used as a cushion in a polyallomer centrifugation tube for use in Beckman SW28 rotor, and the aforementioned mixture of about 20 ml was gently layered over the cushion in such a manner that the layers were not disturbed and the tube was almost filled. The thus prepared tube was subjected to 20 hours of centrifugation at 25,000 rpm and at 20°c. The resulting pellet was washed twice with a small amount of 80% dissolved in TE solution, extracted with phenol/chloroform (1:1) and then subjected to ethanol precipitation with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol. In this way, about 2.5 mg of total RNA was obtained from about 10<sup>8</sup> cells.

Purification of poly (A)<sup>+</sup> RNA from the total RNA was carried out using Oligotex<sup>TM</sup>-dT30 (Super) (manufactured by Japan Synthetic Rubber/Nippon Roche; oligo dT molecules are immobilized on latex particles by covalent bonding, and purification of poly (A)<sup>+</sup> RNA can be attained similar to use of an oligo dT column which is generally used for such purpose). As the result, 20  $\mu$ g of poly (A)<sup>+</sup> RNA was obtained from about 500  $\mu$ g of the total RNA.

### <Example 7>

### Construction of rat cDNA library

Double-stranded cDNA having an EcoRI recognition site at its 5'-end and a Notl recognition site at the 3'-end was synthesized from 5 µg of the poly (A)+ RNA obtained in Example 6, using a Time Saver M cDNA synthesis kit (manufactured by Pharmacia; a cDNA synthesis kit based on the modified method of Okayama and Berg, Mol. Cell. Biol., vol.2, pp.161 - 170, 1982) and DIRECTIONAL CLONING TOOLBOX (manufactured by Pharmacia; a set of a primer 5'-AACTGGAAGAATTCGCGGCCGCAGGAA(T)<sub>18</sub>-3' (SEQ ID NO:15) containing a Noti recognition sequence for use in the synthesis of cDNA and adapters 5'-AATTCGGCACGAG-3' (SEQ ID NO:16) and 5'-CTCGTGCCG-3' (SEQ ID NO:17) for use in the addition of an EcoRI recognition sequence). The thus synthesized cDNA was ligated with 1.2 µg of the expression vector pEF18S (cf. Example 5) which had been digested in advance with EcoRI and Not! and transformed into 8.4 ml of the aforementioned Competent High E. coli DH5 (manufactured by Toyobo Co., Ltd.) As the result, 5.3 x 10<sup>5</sup> transformants were obtained.

#### <Example 8>

Preparation (cloning) of rat TPO cDNA fragment by PCR

The 530,000 clones of the McA-RH8994 cDNA library constructed in Example 7 were cultured overnight in 50 ml of LB medium containing 50 µg/ml of ampicillin, and McA-RH8994 cDNA library plasmids were purified from the resulting cells collected by centrifugation, using QIAGEN-tip100 (manufactured by DIAGEN; an anion exchange silica column for DNA purification use). About 200 µg of plasmid DNA was obtained.

Two anti-sense nucleotide primers AP8-1R and AP8-2R, which correspond to the amino acid sequence of the peptide fragment AP8 described in Example 2, and two sense nucleotide primers  $EF1\alpha-1$  and  $EF1\alpha-2$ , which correspond to the first intron of the human elongation factor  $1\alpha$  of the plasmid vector pEF-18S (cf. Fig. 6) used for the preparation of cDNA library, were synthesized. Synthesis of these primers was effected making use of a 394DNA/RNA Synthesizer (manufactured by Applied Biosystems; a synthesizer based on the β-cyanoethylamidite method) and their purification was effected by the use of an OPC column for synthetic DNA purification use (manufactured by Applied Biosystems; a reverse phase silica gel column for use in the purification of synthetic DNA having trityl groups). Each of the thus synthesized and purified DNA molecules was dissolved in TE solution to a final concentration of 50 µM and stored at -20 c until its use. Synthetic oligonucleotides used in the following procedure were synthesized and purified in the same manner.

The primers AP8-1R and AP8-2R are mixed primers consisting of 17 continued nucleotides in which deoxyinosine is incorporated, as reported by Takahashi *et al.* (*Proc. Natl. Acad. Sci. USA*, vol.82, pp.1931 - 1935, 1985).

The primers EF1 $\alpha$ -1 and EF1 $\alpha$ -2 consisting of 21 and 20 nucleotides, respectively, were synthesized based on the nucleotide sequences of positions 1491 to 1512 and 1513

to 1532 of the genomic sequence reported by Uetsuki et al. (J. Biol. Chem., vol.264, pp.5791 - 5798, 1989).

AP8: Ile	Pro Val	Pro	Pro	Ala	Cys	a Asp	Pro	Arg Le	u I	æu	(SEQ	ID	NO:1	8)
Thr											_			_
Ser											(SEQ	10	NO:1	9)
001						•					(SEQ	ID	NO:20	0),
AP8-1R:	3'-ACG	CTG	GGG	GCI	GAI	GA-5'		(SEQ	ID	NO:	21)			
	A	A	A	T	A	A		(SEQ	ID	NO:	22)			
			T					(SEQ	ID	NO:	23)			
			С					(SEQ	ID	NO:	24)			
AP8-2R:	3'-GGG	GGG	CGG	ACG	CTG	GG-5	)	(SEQ	ID	NO:	25)			
•	A	A	A	A	A			(SEQ	ID	NO:	26)			
	Ŧ	T	T					(SEQ	ID	NO:	27)			
	С	С	С					(SEQ	ID	NO:	28)			

EF1a-1: 5'-GGA TCT TGG TTC ATT CTC AAG-3' (SEQ ID NO:29)
EF1a-2: 5'-CCT CAG ACA GTG GTT CAA AG-3' (SEQ ID NO:30)

Using 3  $\mu$ g of the McA-RH8994 cDNA library plasmid as a template, AP8-1R (500 pmol) and EF1 $\alpha$ -1 (100 pmol) as primers and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.; a set of a thermostable Taql polymerase, a reaction buffer and dNTP for use in PCR), PCR was carried out by GeneAmp<sup>TM</sup> PCR system 9600 (manufactured by Perkin-Elmer; a thermal cycker for PCR) (100  $\mu$ l volume; heating at 95°c for 2 minutes, a total of 35 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 40°c for 1 minute and synthesis at 72°c for 1 minute, and final incubation at 72°c for 7 minutes). In order to improve specificity of the thus amplified DNA fragments, further PCR was carried out (100  $\mu$ l volume; heating at 95°c for 2 minutes,

a total of 35 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 45°c for 1 minute and synthesis at 72°c for 1 minute, and final incubation at 72°c for 7 minutes), using 1  $\mu$ l of the thus obtained PCR reaction solution as a template, and EF1 $\alpha$ -2 (100 pmol) and AP8-2R (500 pmol) as primers.

The thus obtained reaction solution was subjected to 2% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a DNA fragment of about 330 bp as the main product of the PCR which was subsequently purified making use of the aforementioned Prep-A-Gene DNA purification kit (manufactured by Bio-Rad Laboratories, Inc.). Using T4 DNA ligase (manufactured by Life Technologies), the thus purified DNA fragment was subcloned into pCRTMII vector (a vector for the use of TA cloning of PCR products. manufactured by Invitrogen). Since the thermostable polymerase to be used in PCR has a terminal transferase activity, the amplified DNA fragment is directly subcloned into the pCRTMII vector which has a 5'-dT cohesive end, making use of the enzyme's property to add one deoxyadenylic acid to the 3'-end of the PCR-amplified DNA. Plasmid DNA molecules were purified from 28 clones selected at random from the resulting clones using QIAGEN-tip100 (manufactured by DIAGEN) and their nucleotide sequences were determined by a 373A DNA sequencer (a fluorescence sequencer, manufactured by Applied Biosystems), making use of a Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (manufactured by Applied Biosystems; a kit for use in PCR-aided nucleotide sequence determination using fluorescent dyes, based on the dideoxy method of Sanger et al., Proc. Natl. Acad. Sci. USA, vol.74, pp.5463 - 5467, 1977).

When a DNA fragment which encodes three amino acid residues, (Ile/Thr/Ser)-Val-Pro, of the amino acid sequence of AP8 shown above, adjacent to the AP8-2R primer, was selected from the thus obtained DNA fragments and its

whole length was sequenced, it contained cDNA of 261 bp. Position 173 to 175 of this cDNA fragment encoded methionine, and the coding frame coincided with the amino acid frame of AP8. Since the sequence interposing the position 173-175 of the DNA fragment coincided with the sequence of Kozak (Kozak, M., Cell, vol.44, pp.238 - 292, 1986), it seems that this cDNA fragment contains a translation initiation region, coding for the N-terminal of rat TPO protein. This cDNA fragment was named A1. Nucleotide sequence of the A1 fragment excluding the vector sequence, and an amino acid sequence deduced therefrom are shown in the Sequence Listing (SEQ ID NO: 1) attached hereto.

#### <Example 9>

# Screening of rat TPO cDNA clone by PCR

The aforementioned cDNA library was divided into pools of about 10,000 clones, cultured overnight in 1 ml of aforementioned LB medium containing 50 µg/ml of ampicillin and then subjected to plasmid DNA extraction using an automatic plasmid isolation apparatus PI-100 (VER-3.0, manufactured by Kurabo Industries, Ltd.; an automatic plasmid DNA extraction apparatus based on a modified method of the alkali SDS method disclosed in *Molecular Cloning*, Sambrook *et al.*, Cold Spring Harbor Laboratory Press, 1989). Separately from this, the following two oligonucleotides were synthesized based on the cDNA fragment A1 and purified.

- 5' CGAGGGTGTACCTGGGTCCTG 3' (SEQ ID NO:31) (a sense sequence of positions 1 to 17 of the sequence of SEQ ID NO: 1; CGAG represents an adapter sequence)
- 5' CAGAGTTAGTCTTGCGGTGAG 3' (SEQ ID NO:32) (an anti-sense sequence of positions 212 to 232 of the sequence of SEQ ID NO: 1)

Using 1/30 volume of each plasmid DNA sample obtained above as a template, the thus synthesized

oligonucleotides as primers and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.), PCR was carried out by GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) (a total of 30 cycles, each cycle consisting of denaturation at 94°c for 30 seconds, annealing at 66°c for 30 seconds and synthesis at 72 °C for 1 minute). As the result, a specific band of 236 bp was detected in 3 of the 100 pools examined. When one of these 3 pools was divided into sub-pools of about 900 clones to extract plasmid DNA and PCR was carried out in the same manner, a specific band was detected in 3 of the 100 sub-pools tested. When one of these sub-pools was further divided into pools of 40 clones and the same screening process was carried out, the specific band was found in 3 of the 100 pools tested. One of the candidate pools was cultured on an LB plate (LB medium containing 15% agar) supplemented with 50 µg/ml of ampicillin and each of the thus formed colonies was subjected to plasmid DNA extraction and PCR in the same manner. As the result, a positive band was detected in 2 of the 100 clones examined.

#### <Example 10>

#### Sequencing of rat TPO cDNA

Purification of plasmid DNA was carried out basically in accordance with the procedure described in *Molecular Cloning* (Sambrook *et al.*, Cold Spring Harbor Laboratory Press, 1989). Each of the two clones obtained in Example 9 were cultured overnight in 50 ml of LB medium containing 50  $\mu$ g/ml of ampicillin, and about 300  $\mu$ g of plasmid DNA was obtained after purification in the same manner as described in Example 6.

The thus obtained plasmid DNA was subjected to sequencing in the same manner as described in Example 8 using the Taq Dye Deoxy  $^{T\,M}$  Terminater Cycle Sequening Kit

(manufactured by Applied Biosystems) to determine complete nucleotide sequence containing the A1 fragment. As the result, nucleotide sequences of the two clones completely coincided with each other, showing that they are the same clone. One of these clones was selected, and the plasmid carried by this clone was named pEF18S-A2 $\alpha$ . Its nucleotide sequence and an amino acid sequence deduced therefrom are shown in the Sequence Listing (SEQ ID NO: 2).

The sequence shown in the Sequence Listing (SEQ ID NO: 2) has distinct characteristics. The sequence downstream of the 172 bp 5' non-translation region encodes an amino acid sequence rich in hydrophobic amino acids which is presumed to be a protein secretion signal sequence consisting of 21 amino acids starting with methionine. This protein contains 126 amino acid residues, and a 3' non-translation sequence of 1,025 nucleotides and a poly A tail following the termination This protein contains a sequence which codon (TAA). corresponds to the amino acid sequence AP8 analyzed in Example 2 (amino acid numbers 1 to 12 in SEQ ID NO: 2), but does not contain a consensus sequence for N-glycosylation. The 1624-1629 nucleotide sequence located closely to the terminal of the 3' non-translation sequence is different from a consensus sequence but seems to be a potential polyadenylation sequence.

The vector pEF18S-A2 $\alpha$  carried by an *E. coli* strain DH5 has been deposited by the present inventors on February 14, 1994, under the deposit No. FERM BP-4565, in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

<Example 11>
Expression of rat TPO cDNA in COS 1
cells and confirmation of TPO activity

Transfection of plasmid pEF18S-A2α into COS 1 cells was carried out in the following manner by slightly modifying the DEAE-dextran method which includes chloroquine treatment (Sompayrac et al., Proc. Natl. Acad. Sci. USA, vol.78, pp.7575 - 7578, 1981; Luthman et al., Nucl. Acids Res., vol.11, pp.1295 - 1308, 1983). COS 1 cells (ATCC CRL1650) were suspended in the aforementioned DMEM medium containing 10% FCS, put into a 100-mm plastic tissue culture dish and cultured at 37°c in a 5% CO2 incubator until the cells reached a stage of about 40% confluence. Separately, plasmid pEF18S-A2 $\alpha$  (10  $\mu$ g) dissolved in 30  $\mu$ l of HBS (21 mM HEPES. 145 mM NaCl, pH 7.1) was added to 4 ml of the DMEM culture medium which has been supplemented with 500 μg/ml of DEAE-dextran (manufactured by Pharmacia), 80 µM of chloroquine (manufactured by Sigma Chemical Co.), 8% (v/v) of HBS and 9% (v/v) of Nu-Serum (manufactured by Collaborative Research, Inc.). The thus prepared mixture was added to the above cultured COS 1 cells which were washed twice with the DMEM culture medium, and the culturing was continued for 5 hours at 37°c in the 5% CO2 incubator. Thereafter, culture supernatant in the dish was removed by suction, and the remaining cells in the dish were washed twice with the DMEM culture medium, supplemented with 15 ml of the DMEM culture medium containing 10% FCS and cultured at 37°c for 3 to 5 days in the 5% CO2 incubator to recover the resulting culture supernatant.

The thus obtained culture supernatant was extensively dialyzed against IMDM culture medium and evaluated by the rat CFU-MK assay system. TPO activity was found in a dose-dependent fashion in the culture supernatant of COS 1 cells in which the plasmid pEF18S-A2 $\alpha$  was expressed (Fig. 7). Similar to the case of XRP-derived TPO, many megakaryocytes formed elongated cytoplasmic processes on 4

days of culture. By contrast, TPO activity was not found in the culture supernatant of COS 1 cells to which the plasmid pEF18S alone was transfected (Fig. 7). In the M-07e assay system, M-07e cell proliferation enhancing activity was also found in a dose dependent fashion in the culture supernatant of COS 1 cells in which the plasmid pEF18S-A2 $\alpha$  was expressed, but not in the culture supernatant of COS 1 cells in which the plasmid pEF18S alone was expressed. These results demonstrated that A2 $\alpha$  contains a cDNA which encodes a protein having a TPO activity.

Next, a partially purified TPO sample was prepared in order to examine for the ability of this TPO activity to promote platelet production in vivo. Firstly, COS 1 cells transfected with the plasmid pEF18S-A2a were cultured for 3 days in a serum free culture medium which has been supplemented with 0.2 mg of BSA, thereby obtaining about 5.8 liters of serum free culture supernatant. Protease inhibitor p-APMSF was added to the serum-free culture supernatant to the final concentration of 1 mM, and the mixture was filtered using a 0.22 µm filter. To 5,793 ml of the resulting filtrate (protein concentration, 0.229 mg/ml; total protein, 1,326 mg; relative activity, 1,000; total activity, 1,326,000) was added 0.85 moles of NaCl for 1000 ml (288 g in total), thereby obtaining 5,849 ml of solution containing 0.822 M NaCl. The thus prepared solution was applied, at a flow rate of 7 ml/min, to a TSK-gel AF-BLUE 650 MH column (manufactured by Tosoh Corp., catalog No. 08705; 5 cm in diameter and 6 cm in bed height) which has been equilibrated in advance with 20 mM sodium phosphate (pH 7.2) containing 1 M NaCl. completion of the sample application, about 7,900 ml of eluate passed through the column when eluted with 20 mM sodium phosphate (pH 7.2) containing 1 M NaCl. This eluate was pooled and concentrated using an ultrafiltration unit (Omega Ultrasette, nominal molecular weight cutoff of 8,000;

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manufactured by Filtron), thereby obtaining a passed-through fraction F1 (460 ml; protein concentration, 2.11 mg/ml; total protein, 973 mg; relative activity, 16.3). Next, the elution solution was changed to 2 M NaSCN, and the thus eluted TSKgel AF-BLUE 650MH-adsorbed TPO-active fraction F2 (2840 ml) was concentrated to 6.81 ml using an ultrafiltration unit with YM 3 membrane (76 mm in diameter, manufactured by Amicon Corp.). Total protein in this TPO-active fraction F2 was 12.5 mg, and protein yield of F2 at this step was 0.62%. Relative activity of TPO was calculated to be 240. Next, the F2 was applied to a HiLoad 26/60 Superdex 200 pg (manufactured by Pharmacia Biotech, catalog No. 17-1071-01; 2.6 cm in diameter and 60 cm in bed height) and developed with 20 mM sodium acetate (pH 5.5) containing 50 mM NaCl at a flow rate of 1 ml/min. After commencement of the development, TPO activity was found in eluates in the range of from 194 to 260 ml after application. These eluates were pooled to obtain a Superdex 200 pg TPO-active fraction F2 (66 ml; protein concentration, 0.112 mg/ml; total protein, 7.41 mg; relative activity, 142,860; total activity, 1,058,600). buffer A (20 mM sodium acetate, pH 5.5) and buffer B (20 mM sodium phosphate, pH 7.2, containing 500 mM NaCl) were prepared, and the TPO-active fraction was applied at a flow rate of 1 ml/min to a strong cation exchange column RESOURCE S (manufactured by Pharmacia Biotech, catalog No. 17-1178-01: 0.64 cm in diameter and 3 cm in bed height) which has been equilibrated in advance with 100% A. Thereafter, elution was carried out at a flow rate of 0.3 ml/min with 40 minutes of a linear gradient of from 100% A to 100% B. When assayed, the TPO activity was detected in a broad range of eluates (from 5% These TPO-active eluates were pooled and B to 32% B). concentrated using an ultrafiltration unit with YM 3 membrane (25 mm in diameter, manufactured by Amicon Corp.) to obtain a RESOURCE S TPO-active fraction F2 (1.65 ml; protein

concentration, 4.74 mg/ml; total protein, 7.82 mg; relative activity, 71,400; total activity, 558,600).

purified TPO sample The partially subcutaneously administered for consecutive 5 days (474 µg (100 µl)/mouse/day) to ICR male mice (9 weeks old) whose platelet numbers had been measured on the day before the As a control, BSA (200 µg (100 μl)/mouse/day) or a buffer used as the solvent of the partially purified TPO (100 μl/mouse/day) was administered in the same manner. On the next day of the final administration, blood was collected from the heart of each mouse to measure platelet counts using a hemocytometer (F800, manufactured by Toa Ivo Denshi). In the partially purified TPO-administered mice, an increase in platelet counts by a factor of about 2.14 was observed in comparison with platelet counts before the When compared with the control groups, administration. increased platelet counts in the partially purified TPOadministered mice were about 1.74 times higher than those in the BSA-administered mice and about 1.90 higher than those in the buffer-administered mice. These results demonstrated that TPO promotes platelet production in vivo. In addition. since the amount of immunosuppressive acidic protein (IAP), known as a mouse acute phase protein, was not elevated in the partially purified TPO-administered mice, it was strongly suggested that TPO is different from IL-6 and IL-11 with regard to the induction of acute phase protein.

### <Example 12>

#### Detection of TPO mRNA in rat tissues

In order to locate rat TPO mRNA expressing tissue in the rat body, RNA was extracted from various rat tissues. A total of 6 rats were subjected to X-ray irradiation in the same manner as described in Example 1, and various tissues (brain, thymus, lung, liver, heart, spleen, small intestine, kidney,

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testis and bone marrow cells) were excised from the rats on the 11th to 14th day after the X-ray irradiation and immediately frozen in liquid nitrogen. Extraction of total RNA was effected by the use of an RNA isolation reagent ISOGEN (manufactured by Wako Pure Chemical Industries, Ltd.). Amount of ISOGEN to be used was determined according the weight of each frozen tissue sample, and the reagent-added tissue sample was treated with a homogenizer (Physcotron R NS-60, manufactured by NITI-ON Medical & Physical Instruments MFG. Co., LTD) until complete disintegration of the tissue was attained (approximately 45 to 60 seconds at 10,000 rmp). The tissue homogenate was then subjected to total RNA extraction making use of a procedure based on the acid guanidium phenol chloroform method of Chomczynski et al. (Anal. Biochem., vol.162, pp.156 - 159, 1987). As the result, 1.1 to 5.6 mg of total RNA was obtained from respective tissues.

Making use of Oligotex<sup>T M</sup>-dT30 (Super) (manufactured by Japan Synthetic Rubber/Nippon Roche), 20 μg of poly (A)<sup>+</sup> RNA was purified from about 500 μg of total RNA.

Using random primer, first strand of cDNA was synthesized from 1 µg of the poly (A)<sup>+</sup> RNA obtained from each tissue. That is, 1 µg of the poly (A)<sup>+</sup> RNA was dissolved in 10 µl of sterile water, incubated at 70°c for 15 minutes and then rapidly cooled down. To this were added 75 pmoles of random primer (Takara Shuzo Co., Ltd.), 10 U of RNase inhibitor (Boehringer-Mannheim Corp.), 50 mM of Tris-HCl (pH 8.3), 75 mM of KCl, 3 mM of MgCl<sub>2</sub> and 200 U of Super Script<sup>TM</sup> II (a reverse transcriptase manufactured by Life Technologies). The thus prepared solution (total volume, 20 µl) was incubated at 37°c for 1 hour, and the resulting reaction solution was incubated at 70°c for 10 minutes to inactivate the enzyme and stored at -20°c until its use.

New primers for PCR were synthesized based on the rat TPO cDNA sequence obtained in Example 10. Sequences of the thus synthesized primers are as follows.

rTPO-I: 5'-CCT GTC CTG CTG CCT GCT GTG-3' (SEQ ID NO:33) (positions 347 to 367 in SEQ ID NO: 2)

rTPO-N: 5'-TGA AGT TCG TCT CCA ACA ATC-3' (SEQ ID NO:34) (anti-sense primer corresponding to positions 1005 to 1025 in SEQ ID NO: 2)

Using 1/10 volume of each of the synthesized cDNA solution as a template, 1 µM each of the thus synthesized rTPO-I and rTPO-N as primers and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.), PCR was carried out in a volume of 100 µl, making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and heating at 95°c for 2 minutes, repeating a total of 30 cycles, each cycle consisting of denaturation at 95 c for 1 minute, annealing at 57 c for 1 minute and synthesis at 72°c for 1 minute, followed by the final When each of the thus incubation at 72°c for 7 minutes. obtained reaction solutions was subjected to electrophoresis using 2% agarose gel (manufactured by FMC BioProducts) and the amplified bands were examined, bands that seemed to be specific for TPO mRNA were found on gels resulting from the brain, the liver, the small intestines and the kidney. These results indicate that expression of TPO mRNA in rat occurs in tissues of these organs, though its expression quantity cannot be judged. When possible existence of similar expression mode in the human body and the results of Example 4 are taken together into consideration, it seems that the liver is an appropriate starting material for the acquisition of human TPO cDNA.

<Example 13>
Construction of human normal liver-derived cDNA library

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Based on the results of Example 12, the liver was selected as the starting material for use in the cloning of human TPO cDNA. In the same manner as described in Example 7, double-stranded cDNA having an EcoRI recognition site on its 5'-end and a Notl recognition site on its 3'-end was synthesized from 5 µg of commercial normal human liverderived poly (A)+ RNA (manufactured by Clontech; a product extracted based on the acid guanidium phenol chloroform method of Chomczynski et al.), using a Time Saver TM cDNA synthesis kit (manufactured by Pharmacia) and DIRECTIONAL CLONING TOOLBOX (manufactured by Pharmacia). The thus synthesized cDNA was ligated with 1.2 µg of the aforementioned expression vector pEF18S which had been digested in advance with EcoRI and NotI and transformed into 8.4 ml of the aforementioned Competent High E. coli DH5 (manufactured by Toyobo Co., Ltd.) As the result, 1.2 x 10<sup>6</sup> transformants were obtained.

## <Example 14>

# Preparation (cloning) of human TPO cDNA fragment by PCR

Using random primers, first strand of cDNA was synthesized from 1 μg of the commercial normal human liver-derived poly (A)+ RNA (manufactured by Clontech). That is, 1 μg of the poly (A)+ RNA was dissolved in 10 μl of sterile water, incubated at 70 °C for 15 minutes and then rapidly cooled down. To this were added 75 pmoles of random primers (Takara Shuzo Co., Ltd.), 10 U of RNase inhibitor (Boehringer-Mannheim Corp.), 50 mM of Tris-HCl (pH 8.3), 75 mM of KCl, 3 mM of MgCl<sub>2</sub> and 200 U of a reverse transcriptase Super Script<sup>TM</sup> II (manufactured by Life Technologies). The thus prepared solution (total volume, 20 μl) was incubated at 37 °C for 1 hour, and the resulting reaction solution was incubated

at 70°c for 10 minutes to inactivate the enzyme and stored at -20°c until its use.

Primers for PCR use were synthesized based on the rat TPO cDNA sequence (SEQ ID NO: 2). Sequences of the thus synthesized primers are as follows.

rTPO-AIN: 5'-ATG GAG CTG ACT GAT TTG CTC-3' (SEQ ID NO:35) (positions 173 to 193 in SEQ ID NO: 2)

rTPO-N: 5'-TGA AGT TCG TCT CCA ACA ATC-3' (SEQ ID NO:36) (anti-sense primer corresponding to positions 1005 to 1025 in SEQ ID NO: 2)

Using 1/10 volume of the synthesized cDNA solution as a template, 1  $\mu$ M each of the thus synthesized rTPO-AIN and rTPO-N as primers and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.), PCR was carried out with a volume of 100  $\mu$ l, making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and heating at 95°c for 2 minutes, repeating a total of 35 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 40°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes.

The thus obtained reaction solution was subjected to 2% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a DNA fragment of about 620 bp as the main product of the PCR which was subsequently purified making use of the aforementioned Prep-A-Gene DNA purification kit (manufactured by Bio-Rad Laboratories, Inc.). Nucleotide sequence of the thus purified DNA fragment was directly determined by a 373A DNA sequencer (manufactured by Applied Biosystems) making use of the aforementioned Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (manufactured by Applied Biosystems). The thus determined nucleotide sequence excluding the primer moiety and an amino acid sequence

deduced therefrom are shown in the Sequence Listing (SEQ ID NO: 3).

This DNA fragment, excluding the primer sequences, has a length of 580 bp. When compared, the human cDNA showed a homology of 86% with the rat cDNA nucleotide sequence, indicating that this DNA fragment encodes a portion of human TPO cDNA.

## <Example 15>

# Screening of human TPO cDNA clone by PCR

Human TPO-corresponding primers for PCR were synthesized based on SEQ ID NO: 3. Sequences of the thus synthesized primers are as follows.

hTPO-I: 5'-TTG TGA CCT CCG AGT CCT CAG-3' (SEQ ID NO:37) (positions 60 to 80 in SEQ ID NO: 3)

hTPO-J: 5'-TGA CGC AGA GGG TGG ACC CTC-3' (SEQ ID NO:38) (anti-sense primer corresponding to positions 479 to 499 in SEQ ID NO:3)

. The human cDNA library constructed in Example 13 was amplified and divided into pools (each pool containing about 100,000 clones), cultured overnight in 1 ml of the aforementioned LB medium containing 50  $\mu$ g/ml of ampicillin and then subjected to plasmid DNA extraction using an automatic plasmid isolation apparatus PI-100 (VER-3.0, manufactured by Kurabo Industries, Ltd.). The thus extracted DNA was dissolved in TE solution.

Using 5% of the extracted DNA sample as a template, 1  $\mu$ M each of the synthesized oligonucleotides (hTPO-I and hTPO-J) as primers and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.), PCR was carried out in 20  $\mu$ I volume by GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) (a total of 35 cycles, each cycle consisting of denaturation at 95 c for 1 minute, annealing at 59 c for 1

minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes). As the result, a specific band was detected in 3 of the 90 pools used. One of these 3 pools was divided into sub-pools, each containing about 5,000 clones, and plasmid DNA was purified from 90 sub-pools to carry out PCR in the same manner. As the result, specific band was detected in 5 sub-pools. One of these 5 pools was divided into sub-pools, each containing 250 clones, and plasmid DNA was extracted from 90 sub-pools. When these extracted samples were subjected to PCR in the same manner. a specific band was detected in 3 sub-pools. One of these 3 pools was further divided into sub-pools, each containing 30 clones, and plasmid DNA was purified from 90 sub-pools to carry out PCR in the same manner. As the result, a specific band was detected in 3 sub-pools. One of the candidate pools was cultured on the aforementioned LB plate containing 50 μg/ml of ampicillin and each of the thus formed 90 colonies was subjected to plasmid DNA extraction and PCR in the same manner. As the result, a clone HL34 was finally obtained.

#### <Example 16>

# Sequencing of human TPO cDNA

Purification of plasmid DNA was carried out basically in accordance with the procedure described in *Molecular Cloning* (Sambrook *et al.*, Cold Spring Harbor Laboratory Press, 1989). The clone HL34 was cultured overnight in 50 ml of the LB medium containing 50 µg/ml of ampicillin, and the resulting cells collected by centrifugation were suspended in 4 ml of the aforementioned TEG-lysozyme solution. To this were added 8 ml of 0.2 N NaOH/1% SDS solution and then 6 ml of 3 M potassium/5 M acetate solution to suspend the cells thoroughly. After centrifugation of the suspension, the resulting supernatant fluid was treated with phenol/chloroform (1:1), mixed with the same volume of

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isopropanol and then centrifuged. The resulting pellet was dissolved in the TE solution and treated with RNase and then with phenol/chloroform (1:1), followed by ethanol precipitation. The resulting pellet was again dissolved in the TE solution to which were subsequently added NaCl and polyethylene glycol 3,000 to their final concentrations of 0.63 M and 7.5%, respectively. After centrifugation, the pellet was dissolved in the TE solution and precipitated with ethanol. In this way, about 300 µg of the pEF18S-HL34 plasmid DNA was obtained.

The thus purified plasmid DNA was applied to the aforementioned 373A DNA sequencer (manufactured by Applied Biosystems) to determine its complete nucleotide sequence using the aforementioned Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (manufactured by Applied Biosystems). The thus determined nucleotide sequence and an amino acid sequence deduced therefrom are shown in the Sequence Listing (SEQ ID NO: 4). In this case, oligonucleotides synthesized based on the nucleotide sequence of SEQ ID NO: 3 and synthetic oligonucleotides designed based on the internal sequence obtained by the sequence analysis were used in the nucleotide sequence determination as primers.

As the result, it was confirmed that the plasmid clone pEF18S-HL34 comprises a cDNA fragment of 861 bp and contains highly homologous sequences with the amino acid sequences AP8 (amino acid numbers 1 to 12 in SEQ ID NO: 4) and TP2/TP3 (amino acid numbers 157 to 162 in SEQ ID NO: 4) analyzed in Example 2. This DNA fragment seems to encode an open reading frame starting at its position 25, but has no termination codon and contains a poly A tail-like sequence of 76 bases on its 3'-end. Its amino acid sequence, consisting of 253 amino acid residues just before the poly A tail-like sequence, showed 84% homology with the corresponding portion of the rat TPO cDNA (an amino acid sequence moiety

consisting of 147 residues shown in SEQ ID NO: 2). In consequence, this clone was found to be a DNA fragment which encodes a portion of human cDNA that corresponds to rat TPO cDNA. Because of the absence of a termination codon and the presence of a poly A tail-like sequence on its 3'-end, this clone did not seem to be a complete cDNA, but an artificial product which resulted during the procedure of cDNA library construction.

<Example 17>
Expression of human TPO cDNA in COS 1
cells and confirmation of TPO activity

Transfection of the thus obtained plasmid clone pEF18S-HL34 to COS 1 cells was carried out according to the procedure of Example 11. That is, transfection was performed with 10  $\mu g$  of the plasmid DNA by the DEAE-dextran method which includes chloroquine treatment. The COs-1 cells were cultured for 3 to 5 days at 37 °c, and then the supernatant was collected.

The thus obtained culture supernatant was extensively dialyzed against the IMDM culture medium and evaluated by the rat CFU-MK assay system. TPO activity was detected in a dose-dependent fashion in the culture supernatant of COS 1 cells in which a plasmid pEF18S-HL34 was expressed (Fig. 8). After 4 days of culturing, many megakaryocytes formed elongated cytoplasmic processes. In contrast, TPO activity was not found in the culture supernatant of COS 1 cells in which the plasmid pEF18S alone was expressed (Fig. 8). In the M-07e assay system, M-07e cell proliferation enhancing activity was found only in the culture supernatant of COS 1 cells in which the plasmid pEF18S-HL34 was expressed through its transfection. These results demonstrated that pEF18S-HL34 contains a gene which encodes

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a protein having a TPO activity. In addition, it was shown that human TPO acts on rat megakaryocytic progenitor cells (no species specificity).

<Example 18>
Expression of human TPO deletion type
cDNA and confirmation of TPO activity

The clone HL34 obtained in Example 15 contained the cDNA comprising a poly A tail-like continuous sequence on its 3'-side, which did not exist in the rat TPO cDNA and therefore seemed to be an artificial product of the experiment. In consequence, a cDNA molecule from which the poly A tail-like sequence was deleted was prepared in order to see if a protein expressed by the deletion cDNA had a TPO activity. The deletion cDNA was prepared making use of PCR. Sequences of primers used for PCR are as follows in which a restriction enzyme recognition site is added to the 5'-end of each primer (*EcoRl* to hTPO5 and *Notl* and two stop codons TAA and TGA to hTPO3).

hTPO5: 5'-TTT GAA TTC GGC CAG CCA GAC ACC CCG GCC-3' (SEQ ID NO:170) (positions 1 to 21 in SEQ ID NO: 4)

hTPO3: 5'-TTT GCG GCC GCT CAT TAT TCG TGT ATC CTG TTC AGG TAT CC-3' (SEQ ID NO:171) (anti-sense primer corresponding to positions 757 to 780 in SEQ ID NO: 4)

Using 1 µg of plasmid DNA of the plasmid clone pEF18S-HL34 obtained in Example 16 as a template, 10 µM each of the thus synthesized hTPO5 and hTPO3 as primers and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.), PCR was carried out with a volume of 100 µl, making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and repeating a total of 15 cycles, each cycle consisting of denaturation at 95 °c for 1 minute, annealing at 65 °c for 1 minute and

synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes. A band of about 800 bp thus obtained was digested with restriction enzymes EcoRI and NotI, purified and then sub-cloned into the expression vector pEF18S which has been treated in advance with the same restriction enzymes. From the resulting transformants, 5 clones which contained a DNA fragment of about 800 bp were selected to prepare a large quantity of plasmid DNA in accordance with the procedure described in Example 5. The whole length of the amplified region of about 800 bp of each plasmid was subjected to nucleotide sequence analysis to find its complete identity with the nucleotide sequence analyzed in Example 16 (positions 1 to 780 in SEQ ID NO: 4).

The plasmid clone thus obtained was named pHT1-231. The vector pHT1-231 carried by an *E. coli* strain DH5 has been deposited by the present inventors on February 14, 1994 under the deposit No. FERM BP-4564, in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

Transfection of the thus obtained plasmid to COS 1 cells was carried out according to the procedure of Example 11. That is, transfection was performed with 10  $\mu g$  of the plasmid DNA by the DEAE-dextran method which includes chloroquine treatment. The COS1 cells were cultured for 3 to 5 days at 37°c, and then the supernatant was collected.

The thus obtained culture supernatant was extensively dialyzed against the IMDM culture medium and evaluated by the rat CFU-MK assay system. TPO activity was found in the culture supernatant of COS 1 cells in which the plasmid pHT1-231 was expressed (Fig. 9). On 4 days of the culture, many megakaryocytes formed elongated cytoplasmic processes. By contrast, TPO activity was not found in the culture supernatant of COS 1 cells in which the plasmid

pEF18S alone was expressed (Fig. 9). In the M-07e assay system, M-07e cell proliferation enhancing activity was found only in the culture supernatant of COS 1 cells in which the plasmid pHT1-231 was expressed. These results demonstrated that pHT1-231 contains a cDNA which encodes a protein having a TPO activity.

## <Example 19>

# Preparation of the 3'-end region of human TPO cDNA by PCR

The clone HL34 prepared in Example 15 contained the cDNA comprising poly (A) tail-like sequence so that it was suggested that its 3'-end region was incomplete. It was therefore tried to obtain a full-length 3'-end region by PCR. The following four kinds of 5'-side primer for PCR were synthesized based on the sequences determined in Example 16 hTPO-H: 5'-AGC AGA ACC TCT CTA GTC CTC-3' (SEQ ID NO:39) (positions 574 to 594 in SEQ ID NO: 4)

hTPO-K: 5'-ACA CTG AAC GAG CTC CCA AAC-3' (SEQ ID NO:40) (positions 595 to 615 in SEQ ID NO: 4)

hTPO-N: 5'-AAC TAC TGG CTC TGG GCT TCT-3' (SEQ ID NO:41) (positions 660 to 680 in SEQ ID NO: 4)

hTPO-O: 5'-AGG GAT TCA GAG CCA AGA TTC-3' (SEQ ID NO:42) (positions 692 to 712 in SEQ ID NO: 4)

The following 3'-side primers containing mixed nucleotides at the four bases of 3'-end were synthesized in order to amplify the cDNA from the beginning of the poly (A) portion. As anchor primer, without the mixed nucleotide, was also synthesized.

hTPO3mix: 5'-TAG CGG CCG C(T) <sub>17</sub> G GGG-3'	(SEQ ID NO:43)			
A AAA-3'	(SEQ ID NO:44)			
C TTT-3'	(SEQ ID NO:45)			
CCC-3'	(SEQ ID NO:46)			
hTPO3anchor: 5'-TAG CGG CCG C(T)++-3'	(SEQ ID NO:47)			

The first strand of cDNA was synthesized from 1 μg of the commercial normal human liver-derived poly (A)+ RNA (manufactured by Clontech), such as in Example 14, but using 0.5 µg of oligo dT primer (it is included in TimeSaver<sup>TM</sup>cDNA Synthesis Kit manufactured by Pharmacia). Using 1/10 volume of the synthesized cDNA solution as a template, 20 μM of the hTPO-H primer and 10μM of the hTPO3mix primer and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaqTM DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.), PCR was carried out with volume of 50 µl, making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and heating at 96°c for 2 minutes, repeating a total of 10 cycles, each cycle consisting of heat denaturation at 96°c for 1 minute, annealing at 48°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes.

The second PCR was carried out by using 1/10 volume of the first PCR resulting solution as a template, 20  $\mu$ M of the hTPO-K primer and 10 $\mu$ M of the hTPO3mix primer with volume of 50  $\mu$ l, heating at 96°C for 2 minutes, repeating a total of 10 cycles, each cycle consisting of heat denaturation at 96°C for 1 minute, annealing at 63°C for 1 minute and synthesis at 72°C for 1 minute, followed by the final incubation at 72°C for 7 minutes.

The third PCR was carried out by using 1/10 volume of the second PCR resulting solution as a template, 20  $\mu$ M of the hTPO-N primer and 10 $\mu$ M of the hTPO3mix primer with volume of 50  $\mu$ l, heating at 96°c for 2 minutes, repeating a total of 10 cycles, each cycle consisting of heat denaturation at 96°c for 1 minute, annealing at 63°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes.

The fourth PCR was carried out by using 1/10 volume of the third PCR resulting solution as a template, 20

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 $\mu M$  of the hTPO-O primer and 10  $\mu M$  of the hTPO3mix primer with volume of 50  $\mu l$ , heat at 96°c for 2 minutes, repeating a total of 10 cycles, each cycle consisting of heat denaturation at 96°c for 1 minute, annealing at 63°c for 1 minute and synthesis at 72°c for 1 minute, followed by a final incubation at 72°c for 7 minutes.

The fifth PCR was carried out by using 1/10 volume of the fourth PCR resulting solution as a template, 20  $\mu$ M of the hTPO-O primer and 20 $\mu$ M of the hTPO3anchor primer with volume of 50  $\mu$ l, heating at 96°C for 2 minutes, repeating a total of 10 cycles, each cycle consisting of heating denaturation at 96°C for 1 minute, annealing at 58°C for 1 minute and synthesis at 72°C for 1 minute, followed by the final incubation at 72°C for 7 minutes.

The thus obtained reaction solution was subjected to 2% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a DNA fragment of about 600 bp as the main product of the PCR which was subsequently purified making use of the aforementioned Prep-A-Gene DNA purification kit (manufactured by Bio-Rad Laboratories, Inc.). Nucleotide sequence of the thus purified DNA fragment was directly determined by a 373A DNA sequencer (manufactured by Applied Biosystems) making use of the aforementioned Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (manufactured by Applied Biosystems). The thus determined nucleotide sequence and an amino acid sequence deduced therefrom are shown in the Sequence Listing (SEQ ID NO: 5).

This DNA fragment has a nucleotide sequence coding for 130 amino acids starting with the primer hTPO-O, followed by sequence of more than 180 nucleotides at 3'-end (the nucleotides after 577 position could not determined). The 30 amino acid sequence starting with glycine coincides with the amino acid sequence of the positions 203 to 232 in SEQ ID NO: 4. The nucleotide sequence of positions 1 to 94 also

coincides with the nucleotide sequence of the positions 692 to 785 in SEQ ID NO: 4.

As the result of sequence analysis of the cDNA fragment in Example 17 and PCR-amplified fragment in the present Example, it is reasonably estimated that human TPO protein consists of 353 amino acids containing 21 amino-acid signal sequence, as shown in SEQ ID NO: 6.

## <Example 20>

# Reconstruction of human normal liver-derived cDNA library

Since the clone HL34 obtained in Example 15 contained the poly A tail-like sequence directly on its 3'-end of its open reading frame without termination codons and seemed therefore to be an artificial product of the cDNA synthesis, a cDNA library was reconstructed using 5 μg of a commercial normal human liver-derived poly (A)+ RNA preparation (manufactured by Clontech). Synthesis of cDNA was carried out using SuperScript<sup>TM</sup> Lambda System for cDNA Synthesis and  $\lambda$  Cloning Kit and SuperScript TM II RNase H (both manufactured by LIFE TECHNOLOGIES). The poly (A)+ RNA was subjected to heat denaturation and then added to 20 µl of a reaction solution containing a Notl sequence-included oligo dT as a primer attached to the kit (50 mM Tris-HCl, pH 8.3, 75 mM KCI, 3 mM MgCl2, 1 mM DTT, 1 mM dNTP mix, 200 U SuperScript TM II RNase H-), followed by 60 minutes of incubation at 37°c. After synthesis of the second strand of cDNA (2 hours of incubation at 16 c in 150 µl of a reaction solution containing 25 mM Tris-HCl, pH 8.3, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 250 μM dNTP mix, 5 mM DTT, 40 U of E. coli DNA polymerase I, 2 U of E. coli RNase H and 10 U of E. coli DNA ligase), 10 U of T4 DNA polymerase was added and the resulting mixture was incubated at 16°c for 5 minutes. The reaction solution was heated at 65°c for 10 minutes and extracted with the same volume of phenol/chloroform, and cDNA molecules having a length of less than 400 bp were removed using SizeSep<sup>TM</sup> 400 spun column (a spun column for the removal of low molecular weight DNA, attached to TimeSaver<sup>TM</sup> cDNA Synthesis Kit manufactured by Pharmacia). After the addition of EcoRI Adaptor (attached to Directional Cloning Toolbox manufactured by Pharmacia), the thus treated sample was digested with NotI and again applied to SizeSep<sup>TM</sup> 400 spun column to remove low molecular weight DNA. The thus synthesized 1.3  $\mu g$  of the double-stranded cDNA having an EcoRI recognition sequence on its 5'-end and a NotI recognition sequence on its 3'-end was ligated with the expression vector pEF18S which had been previously digested with EcoRI and Notl, and then transformed into 9.2 ml of Competent High E. coli DH5 (manufactured by Toyobo Co., Ltd.) The thus obtained human liver cDNA library (hTPO-F1) contained 1.0 x 106 transformants.

<Example 21>

Screening of TPO cDNA clone from human liver cDNA library hTPO-F1

Human TPO cDNA-corresponding primers for PCR were synthesized based on the Sequence ID Nos. 3 and 6 shown in the SEQUENCE LISTING. Sequences of the thus synthesized primers are as follows.

hTPO-I: 5'-TTG TGA CCT CCG AGT CCT CAG-3' (SEQ ID NO:48) (positions 60 to 80 in SEQ ID NO: 3)

hTPO-KU: 5'-AGG ATG GGT TGG GGA AGG AGA-3' (SEQ ID NO:49) (anti-sense primer corresponding to the sequence of positions 901 to 921 in SEQ ID NO: 6)

The human liver cDNA library hTPO-F1 (1.0 x 10<sup>6</sup> transformants) constructed in Example 20 was divided into 3 pools (pool # 1 - 3) and the pools were frozen. PCR was

carried out using 2 µg of the plasmid DNA prepared from each pool as a template and 1 µM each of the synthesized oligonucleotides (hTPO-I and hTPO-KU) as primers. GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo Co., Ltd.), PCR was performed in 100 µl volume by GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) (a total of 35 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 59°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes). As the result, a DNA fragment having the expected size was amplified when the plasmid DNA prepared from the pool # 3 was used. The # 3 pool was then divided into subpools, each containing 15,000 transformants, and these subpools were cultured overnight in 1 ml of LB medium containing 50 ug/ml of ampicillin, followed by extraction of plasmid DNA using an automatic plasmid isolation apparatus PI-100. The thus extracted DNA was dissolved in TE solution, and 5% of the resulting solutions were used as templates to carry out PCR using the same primers and under the same conditions described above. As the result, amplification of DNA having the expected size was found in 6 of the 90 pools. When one of these positive pools was divided into subpools, each containing 1.000 clones, and plasmid DNA was extracted and PCR was carried out in the same manner as described above, DNA amplification was not observed. The density of bands on electrophoresis gels of DNA fragments amplified by a series of PCR became thinner as the subpool was further subdivided, which seemed to be caused by a low recovery of the plasmid DNA due to poor growth of the clone of interest. Therefore, another screening was carried out by means of colony hybridization using the original # 3 pool.

The # 3 pool was spread on 100 LB agar plates of 15 cm in diameter in such an inoculum size that 4,100 colonies

grew on each LB agar plate. After preparing a replica plate from each of the thus inoculated plates, one of the duplicate plates was cultured at 37 c for 6 hours to recover colonies grown on the plate and to extract the plasmid DNA samples. When PCR of these DNA samples was carried out in the same manner as described above, amplification of a band equivalent to the expected size was observed in one of 100 subpools. Two replica filters were prepared from the plate of this subpool using BIODYNE<sup>TM</sup> A TRANSFER MEMBRANE (manufactured by PALL). Denaturation of the filters were performed by soaking them in 10% SDS for 10 minutes, in 0.5 N NaOH/1.5 M NaCl for 10 minutes and in 0.5 M Tris-HCl (pH 8.0)/1.5 M NaCl for 10 minutes in that order, followed by 30 minutes of air-drying and subsequent 1 hour of baking at 80°c in a vacuum oven. The thus baked filters were washed with 6 x SSC (prepared by diluting 20 x SSC stock solution consisting of 175.3 g of NaCl and 88.2 g of Na citrate dissolved in 1 liter water, pH 7.0) Prehybridization of the thus supplemented with 1% SDS. washed filters were carried out by incubating it at 42°c for 30 minutes with shaking in 30 ml of a reaction solution consisting of 50% formamide, 5 x SSC, 5 x Denhardt's solution (prepared from 50 x Denhardt's solution containing 5 g of Ficoll, 5 g of polyvinylpyrrolidone and 5 g of bovine serum albumin fraction V in 500 ml water), 1% SDS and 20 µg/ml of After prehybridization, the reaction salmon sperm DNA. solution was exchanged with 30 ml of a hybridization solution having the same composition, mixed with a probe which had been labeled with  $[\alpha^{-32}P]$  dCTP (manufactured by Amersham) and then incubated with shaking at 42°c for 20 hours. The labeled probe used in this experiment was the EcoRI/BamHI fragment of the plasmid pEF18S-HL34, which was obtained by purifying a portion of SEQ ID NO: 4 ranging from its 5'-end to the 458 position base and by labeling the purified portion by

random primer technique with Megaprime DNA Labelling System (a kit manufactured by Amersham based on the method disclosed in Anal. Biochem., 132, 6 - 13, 1983). The thus treated filters were washed in 2 x SSC/0.1% SDS solution at 42°c for 30 minutes and then in 0.2 x SSC/0.1% SDS solution at 42°C for 30 minutes. The filters were then subjected to 16 hours of autoradiography at -70°c using an intensifying screen and an X-OMAT<sup>TM</sup> AR5 film (manufactured by Eastman Kodak). As the result, a single signal considered to be positive was observed. Colonies approximately corresponding to this signal were collected from the original plate and inoculated again onto a 10-cm LB agar plate. A total of 50 colonies grown on the plate were separately cultured, and their DNA samples were subjected to PCR using the aforementioned primers hTPO-I and hTPO-KU under the same conditions as described above. As the result, amplification of a band equivalent to the expected size was found in only one clone, which was designated pHTF1.

# <Example 22> <u>Determination of nucleotide sequence of human TPO cDNA clone</u> pHTF1

Purification of plasmid DNA was carried out basically according to the procedure described in *Molecular Cloning* (Sambrook *et al.*, Cold Spring Harbor Laboratory Press, 1989). The clone pHTF1 was cultured overnight in 50 ml of the LB medium containing 50 µg/ml of ampicillin. The resulting cells were collected by centrifugation and suspended in 4 ml of the aforementioned TEG-lysozyme solution. To this were added 8 ml of 0.2 N NaOH/1% SDS solution and then 6 ml of 3 M potassium/5 M acetate solution to suspend the cells thoroughly. After centrifugation of the suspension, the resulting supernatant was treated with phenol/chloroform (1:1), mixed with the same volume of isopropanol and then

centrifuged. The resulting pellet was dissolved in TE solution and treated with RNase and then with phenol/chloroform (1:1), followed by ethanol precipitation. The resulting pellet was again dissolved in TE solution to which were subsequently added NaCl and polyethylene glycol 3,000 to their final concentrations of 0.63 M and 7.5%, respectively. After centrifugation, the pellet was dissolved in TE solution and precipitated with ethanol. In this way, about 300  $\mu g$  of the plasmid DNA pHTF1 was obtained.

The thus purified plasmid DNA was applied to the aforementioned 373A DNA sequencer (manufactured by Applied Biosystems) to determine its complete nucleotide sequence using the aforementioned Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (manufactured by Applied Biosystems). The thus determined nucleotide sequence and the deduced amino acid sequence are shown in the Sequence Listing (SEQ ID NO: 7). Oligonucleotides synthesized based on the nucleotide sequence of SEQ ID NO: 6 and on the internal sequence obtained by their sequence reaction analysis were used in the nucleotide sequence determination as primers.

As the result, it was confirmed that the clone pHTF1 contains a cDNA fragment of 1,721 bp and has high homology with the amino acid sequences AP8 (amino acid numbers 1 to 12 in SEQ ID NO: 7) and TP2/TP3 (amino acid numbers 157 to 162 in SEQ ID NO: 7) analyzed in Example 2. This DNA fragment seems to have a 5' noncoding region of 101 bases, an open reading frame consisting of 353 amino acid residues, starting at a methionine residue encoded at the 102 to 104 nucleotide positions and ending at a glycine residue encoded at the 1,158 to 1,160 nucleotide positions, the subsequent termination codon (TAA), a 3' noncoding region of 531 bases and a poly A tail sequence of 30 bases. The amino acid sequence of a protein considered to be encoded by the open reading frame completely coincided with the predicted amino

acid sequence of human TPO shown in SEQ ID NO: 6. The cDNA sequence of pHTF1 has a larger size than the putative cDNA sequence shown in SEQ ID NO: 6, and contains 77 more additional bases at the 5' side and 347 more additional bases at the 3' side in front of its poly A tail sequence. Also, the nucleotide sequence was different from SEQ ID NO: 6 at 3 positions. That is, A (position 84), A (position 740) and G (position 1,198) in SEQ ID NO: 7 were C, T and A in SEQ ID NO: 6. respectively. Only the mutation at position 740 was included in the protein-encoding region, but this mutation did not cause amino acid exchange because both bases A and T were the third base of threonine codons. Although the cause of these base substitutions was not clear at that time, analysis of the plasmid clone pHTF1 confirmed that human TPO protein comprised 353 amino acid residues having a 21 amino acid Molecular weight of the mature protein signal sequence. excluding the signal sequence was estimated to be 35,466.

The vector pHTF1 carried by an *E. coli* strain DH5 has been deposited by the present inventors on March 24, 1994 under the accession No. FERM BP-4617, at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

<Example 23>
Expression of human TPO cDNA clone pHTF1 in COS 1 cells and confirmation of TPO activity

Transfection to COS 1 cells of the thus obtained plasmid clone pHTF1 was carried out according to the procedure of Example 11. That is, transfection was performed with 10  $\mu g$  of the plasmid DNA by the DEAE-dextran method which includes chloroquine treatment. The transfected COS1

cells were cultured for 3 days at 37°c, and the culture supernatant was collected.

The culture supernatant was extensively dialyzed against the IMDM culture medium and evaluated by the rat CFU-MK assay system. TPO activity was detected in a dose-dependent fashion in the supernatant of COS1 cells in which the plasmid pHTF1 was expressed (Fig. 10a). By contrast, TPO activity was not found in the culture supernatant of COS1 cells in which the plasmid pEF18S alone was expressed (Fig. 10a). Similar results were obtained in the M-07e assay system. The supernatant of COS1 cells, in which the plasmid pHTF1 was expressed, significantly augmented M-07e cell proliferation in a dose-dependent manner (Fig. 10b). These results demonstrated that pHTF1 contains a gene which encodes a protein having TPO activity.

## <Example 24>

# Cloning of human TPO chromosomal DNA

. Cloning of human TPO chromosomal DNA was carried out using human TPO cDNA as a probe. A genomic library used in the cloning was a gift from Prof. T. Yamamoto at the Gene Research Center, Tohoku University (the library reported by Sakai et al. in J. Biol. Chem., 269, 2173 - 2182, 1994, constructed by partially digesting human chromosomal DNA with a restriction enzyme Sau3Al and ligating the partial digest to the BamHI site of a phage vector Lambda EMBL3 manufactured by Stratagene). Screening from this library using human TPO cDNA as a probe was carried out basically according to the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). Using E. coli LE392 as a host, the library was inoculated onto a 15-cm plate containing NZYM (10 g of NZ amine, 5 g of NaCl, 5 g of Bacto Yeast Extract, 2 g of MgSO<sub>4</sub>.7H<sub>2</sub>O and 15 g of agar in 1 liter water, pH 7.0) in such an inoculum size that one plate

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contained 30,000 phage particles. Two replica filters were prepared from each of the thus prepared 18 plates using BIODYNE<sup>TM</sup> A TRANSFER MEMBRANE (manufactured by PALL). Denaturation of the filters was performed by soaking them in 0.5 N NaOH/1.5 M NaCl for 10 minutes and then in 0.5 M Tris-HCI (pH 8.0)/1.5 M NaCI for 10 minutes, followed by 30 minutes of air-drying and subsequent 1.5 hours of baking at 80°c in a vacuum oven. Prehybridization was carried out by incubating the thus treated filters at 42°c for 1 hour in 500 ml of a reaction solution consisting of 50% formamide, 5 x SSC, 5 x Denhardt's solution, 1% SDS and 20 µg/ml of salmon sperm DNA. For use as a probe, a human TPO cDNA fragment (base position numbers 178 to 1,025 in SEQ ID NO: 7) was amplified by PCR and purified, and the purified fragment was labeled with 32P making use of Random Primer DNA Labelling Kit (a DNA labeling kit manufactured by Takara Shuzo based on the random primer method disclosed in Anal. Biochem., 132, 6 - 13, 1983). Sequences of primers used in this PCR are as follows. hTPO-I: 5'-TTG TGA CCT CCG AGT CCT CAG-3' (SEQ ID NO:50) (positions 178 to 198 in SEQ ID NO: 7) htpo-n: 5'-AGG GAA GAG CGT ATA CTG TCC-3' (SEQ ID NO:51) (anti-sense primer corresponding to the sequence of positions

Using the isotope-labeled probe, hybridization was carried out at 42°c for 20 hours in 500 ml of a reaction solution having the same composition of the prehybridization solution. The resulting filters were washed 3 times in 2 x SSC/0.1% SDS solution at room temperature for 5 minutes, and then once in 0.1 x SSC/0.1% SDS solution at 68°c for 1 hour. The filters were then subjected to 16 hours of autoradiography at -70°c using an intensifying screen and an X-OMATTM AR5 film (manufactured by Eastman Kodak). As the result, 13 positive signals were obtained. Plaques approximately corresponding to each of the positive signals were collected

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from the original plates and inoculated again onto 15-cm NZYM plates in such an inoculum size that 1,000 plaques were formed on each plate. Two replica filters were prepared from each of the resulting plates to carry out hybridization under the same conditions described above. As the result, positive signals were detected on all filters of the 13 groups. A single plaque was recovered from each of the resulting plates to prepare phage DNA by the plate lysate method described in *Molecular Cloning*. Phage DNA samples thus prepared from the 13 clones were checked for the presence of the cDNA coding region, by PCR using primers having the following sequences.

hTPO-L: 5'-GGC CAG CCA GAC ACC CCG GCC-3' (SEQ ID NO:52) (positions 1 to 21 in SEQ ID NO: 6)

hTPO-F: 5'-ATG GGA GTC ACG AAG CAG TTT-3' (SEQ ID NO:53) (anti-sense primer corresponding to the sequence of positions 127 to 147 in SEQ ID NO: 6)

hTPO-P: 5'-TGC GTT TCC TGA TGC TTG TAG-3' (SEQ ID NO:54) (positions 503 to 523 in SEQ ID NO: 6)

hTPO-V: 5'-AAC CTT ACC CTT CCT GAG ACA-3' (SEQ ID NO:55) (anti-sense primer corresponding to the sequence of positions 1,070 to 1,090 in SEQ ID NO: 6)

When PCR was carried out using these primer combinations, 5 of the 13 clones seemed to contain entire amino acid coding region predicted from the cDNA. Chromosomal DNA molecules contained in these 5 clones had a similar length of about 20 kb and showed almost the same pattern when a preliminary restriction enzyme analysis was performed. Therefore, one of these clones (clone  $\lambda$ HGT1) was selected and analyzed by Southern blotting. That is, 1  $\mu$ g of DNA of the clone  $\lambda$ HGT1 was digested completely with a restriction enzyme EcoRI or HindIII and applyeded to 0.8% agarose get electrophoresis, and bands on the get were transferred on BIODYNETM A TRANSFER MEMBRANE (manufactured by PALL). The resulting filter was air-dried for

30 minutes and subjected to 2 hours of baking at 80 c in a vacuum oven. Prehybridization was performed by incubating the thus treated filter at 42°c for 1 hour in 50 ml of a reaction solution consisting of 50% formamide, 5 x SSC, 5 x Denhardt's solution, 1% SDS and 20 µg/ml of salmon sperm DNA. For use as a probe, a human TPO cDNA fragment (base position numbers 178 to 1,025 in SEQ ID NO: 7) was amplified by PCR and purified, and the purified fragment was labeled with 32P using Random Primer DNA Labelling Kit (manufactured by Takara Shuzo). Using the isotope-labeled probe, hybridization was carried out at 42°c for 20 hours in 50 ml of a reaction solution having the same composition of the The resulting filter was washed 3 prehybridization solution. times in 2 x SSC/0.1% SDS solution at room temperature for 5 minutes, and then once in 0.1 x SSC/0.1% SDS solution at 68°c The filter was then subjected to 16 hours of autoradiography at -70°c using an intensifying screen and an X-OMAT<sup>TM</sup> AR5 film (manufactured by Eastman Kodak). As the result, a single band of about 10 kb was observed in the case of the HindIII digestion. Therefore, 10 µg of DNA of the clone λHGT1 was digested with HindIII and subjected to 0.8% agarose gel electrophoresis, and the 10 kb band was excised from the gel, purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and subcloned into a cloning vector pUC13 (manufactured by Pharmacia) which had been previously digested with Hindll. In this case, Competent-High E. coli DH5 manufactured by TOYOBO was used as the host strain.

Of the thus obtained clones, a clone containing the 10 kb HindIII fragment was selected and designeted pHGT1.

A restriction map of phage clone  $\lambda$ HGT1 is shown in Fig.11.

<Example 25>
Determination of nucleotide sequence

# of human TPO chromosomal clone pHGT1

Culturing of the clone pHGT1 and purification of plasmid DNA were carried out basically according to the method described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). The clone pHGT1 was cultured overnight in 50 ml of the LB medium containing 50 The resulting cells were collected by μg/ml of ampicillin. centrifugation and suspended in 4 ml of the aforementioned TEG-lysozyme solution. To this were added 8 ml of 0.2 N NaOH/1% SDS solution and then 6 ml of 3 M potassium/5 M acetate solution to suspend the cells thoroughly. centrifugation of the suspension, the resulting supernatant was treated with phenol/chloroform (1:1), mixed with the same volume of isopropanol and then centrifuged. resulting pellet was dissolved in the TE solution and treated with RNase and then with phenol/chloroform (1:1), followed by ethanol precipitation. The resulting pellet was again dissolved in the TE solution to which were subsequently added NaCl and polyethylene glycol 3,000 to their final concentrations of 0.63 M and 7.5%, respectively. After centrifugation, the pellet was dissolved in the TE solution and precipitated with ethanol. In this way, about 300 µg of the plasmid DNA pHGT1 was obtained.

Using the aforementioned Taq Dye DeoxyT M Terminater Cycle Sequening Kit (manufactured by Applied Biosystems), the thus purified plasmid DNA was applied to the aforementioned 373A DNA sequencer (manufactured by Applied Biosystems) to determine its nucleotide sequence around the protein-encoding region predicted from the cDNA nucleotide sequence. The thus determined nucleotide sequence and the deduced amino acid sequence are shown in the Sequence Listing (SEQ ID NO: 8). In this case, oligonucleotides used in Example 22 for the nucleotide sequence analysis of cDNA and synthetic

oligonucleotides designed based on the internal sequence obtained by their sequence reaction analysis were used as primers in the nucleotide sequence determination.

As the result, chromosomal DNA carried by the plasmid clone pHGT1 was found to contain an entire coding region of the amino acid sequence deduced from SEQ ID NO: 6, and nucleotide sequence of the coding region coincided completely with that of SEQ ID NO: 6. In addition, a region corresponding to the amino acid-encoding exon contained 4 introns having lengths of 231 bp, 286 bp, 1,932 bp and 236 bp in that order counting from the 5' side (Fig. 11). Also, the nucleotide sequence was different from the cDNA nucleotide sequence of SEQ ID NO: 7 at 3 positions which were the same as the positions described in Example 22 (different positions between Sequence ID Nos. 6 and 7). That is, A (position 84), A (position 740) and G (position 1,198) in SEQ ID NO: 7 was respectively C, T and A in SEQ ID NO: 8. Thus, it was revealed that the nucleotide sequence of the human TPO cDNA clone pHTF1 obtained in Example 21 is different from the nucleotide sequence of the human chromosomal DNA clone pHGT1 at 3 positions. Therefore, in order to analyze if these mutations in the cDNA clone pHTF1 sequence reflect the chromosomal DNA sequence, nucleotide sequences of other 4 clones among the 5 chromosomal DNA clones independently selected by the screening were determined. The sequence analysis was carried out by a direct nucleotide sequence determination method using a phage DNA sample prepared according to the plate lysate method described in Molecular Cloning. Sequencing of each clone was performed by applying it to the aforementioned 373A DNA sequencer (manufactured by Applied Biosystems), using the sequence primers used in Example 22, which have been synthesized based on such sequence portions that the aforementioned 3 mutation positions in the nucleotide sequence could be analyzed, and the aforementioned Taq Dye

Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (manufactured by Applied Biosystems). It was found that nucleotide sequences of all 4 clones were identical to that of SEQ ID NO: 6. The positions 84 and 740 corresponding to SEQ ID NO: 7 were substituted by C and T, respectively, in these 4 clones. But the position 1,198 was G in 2 clones and A in other 2 clones. In other words, it was revealed that there are two types of nucleotide sequences inherent to the original chromosomal DNA. At present, it is not clear if such differences in the nucleotide sequence are derived from homologous chromosome or from plural genes. In addition, it is suggested that the differences in the nucleotide sequence may be due to a racial difference, because the poly (A)+ RNA purchased from Clontech is a Caucasian origin while the chromosomal DNA is a Japanese origin.

The vector pHGT1 carried by an *E. coli* strain DH5 has been deposited by the present inventors on March 24, 1994 under the accession No. FERM BP-4616, at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

<Example 26>
Expression of human TPO chromosomal DNA in COS 1 cells and confirmation of TPO activity

The plasmid clone pHGT1 obtained by the subcloning contained a total of 4 EcoRI recognition sequences, 3 in the insert moiety and 1 in the vector. The nucleotide sequence analysis revealed that the entire human TPO protein-encoding region was included in a DNA fragment of about 4.3 kbp which was interposed between the EcoRI recognition sequence closest to the 5' side in the insert and the EcoRI recognition sequence in the vector. Therefore, this fragment was ligated

with an EcoRI-treated expression vector pEF18S, and obtained 4 human TPO expression plasmid pEFHGTE#1-4 (see Fig. 11). By preparing these plasmid DNA, an expression experiment was carried out. Purification of plasmid DNA was carried out basically according to the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989), thereby obtaining about 250 µg of the plasmid DNA.

Transfection to COS 1 cells of the thus obtained clones pEFHGTE#1-4 was carried out according to the procedure of Example 11. That is, transfection was performed with 10  $\mu g$  of the plasmid DNA by the DEAE-dextran method which includes chloroquine treatment. The transfected COS1 cells were incubated for 3 days at 37 c and then the culture supernatants were collected.

The culture supernatants were extensively dialyzed against the IMDM culture medium and evaluated by the rat CFU-MK assay system. TPO activity was detected in a dose-dependent fashion in the supernatant of COS1 cells in which each of four clones (pEFHGTE#1-4) was expressed. By contrast, TPO activity was not found in the supernatant of COS1 cells in which the plasmid pEF18S alone was expressed. Representative data with pEFHGTE#1 is shown in Fig. 12a. Similar results were obtained in the M-07e assay system. The supernatant of COS1 cells, in which each of four clones (pEFHGTE#1-4) was expressed, significantly augmented M-07e cell growth in a dose-dependent fashion. Representative data with pEFHGTE#1 is shown in Fig. 12b.

These results demonstrated that the plasmid clones pEFHGTE#1-4 carry the functional chromosomal DNA of human TPO.

<Example 27>
Preparation of human TPO deletion type DNA and its
expression in COS 1 cells and confirmation of TPO activity

The results in Example 18 indicated that human TPO could exhibit its biological activity even after the Thus, in order to further removal of its carboxyl third. evaluate the biologically active portions, deletion derivative experiments were performed. In this Example, TPO deletion derivatives lacking 20, 40, 60 or 68 amino acids from the carboxyl-terminal end of the TPO protein (amino acids 1-231) encoded by pHT1-231 were examined for their ability to exert The shortest derivative TPO in vitro biological activity. (amino acids 1-163) still included amino acid sequences corresponding to TP2/3 of rat plasma TPO described in Example 2. The deletion plasmids were prepared by PCR using the DNA of the plasmid clone pHT1-231 obtained in Example 18 as template and synthesized oligonucleotides as primers. Sequences of primers used for PCR were as follows:

hTPO-5: 5'-TTT GAA TTC GGC CAG CCA GAC ACC CCG GCC-3' (SEQ ID NO:56) (prepared by adding an *EcoRI* recognition sequence to the sequence of positions 1 to 21 in SEQ ID NO:4; this is the identical sequence described in Fig.9);

hTPO-S: 5'-TTT GCG GCC GCT CAT TAG CTG GGG ACA GCT GTG GTG GGT-3' (SEQ ID NO:57) (an antisense primer corresponding to the sequence of positions 555 to 576 in SEQ ID NO:4, prepared by adding two termination codons TAA and TGA and a Not1 recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 163 amino acid residues);

hTPO-4: 5'-TTT GCG GCC GCT CAT TAC AGT GTG AGG ACT AGA GAG GTT CTG-3' (SEQ ID NO:58) (an antisense primer corresponding to the sequence of positions 576 to 600 in SEQ ID NO:4, prepared by adding two termination codons TAA and TGA and a *Not*I recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 171 amino acid residues);

hTPO-30: 5'-TTT GCG GCC GCT CAT TAT CTG GCT GAG GCA GTG AAG TTT GTC-3' (SEQ ID NO:59) (an antisense primer corresponding to the sequence of positions 636 to 660 in SEQ ID NO:4, prepared by adding two termination codons TAA and TGA and a *Not*I recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 191 amino acid residues); and

hTPO-2: 5'-TTT GCG GCC GCT CAT TAC AGA CCA GGA ATC TTG GCT CTG AAT-3' (SEQ ID NO:60) (an antisense primer corresponding to the sequence of positions 696 to 720 in SEQ ID NO:4, prepared by adding two termination codons TAA and TGA and a *Not*l recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 211 amino acid residues).

Using 1 µg of plasmid DNA of the clone pHT1-231 obtained in Example 18 as a template, 10  $\mu M$  each of the thus synthesized hTPO-5 (for 5' side) and hTPO-2, -3, -4 and -S (for 3' side) as primers and GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), PCR was carried out with a volume of 100 µl, using GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and repeating a total of 20 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 65 °C for 1 minute and synthesis at 72 °C for 1 minute. followed by the final incubation at 72°c for 7 minutes. A band of interest thus obtained by each PCR was digested with restriction enzymes EcoRI and NotI and the resulting digest was subjected to 1% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a main DNA fragment having an expected size amplified by each PCR. isolated DNA fragment was purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and then subcloned into the expression vector pEF18S which had been previously treated with the same restriction enzymes. In this case, Competent-High E. coli DH5 manufactured by TOYOBO was used as the host strain. From the resulting transformants which originated from each PCR, 4 to 5 clones containing the insert of expected size were selected to prepare plasmid DNA samples basically according to the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). In a series of such operations. plasmid DNA samples were obtained from a deletion derivative coding for positions 1 to 163 amino acid residues (pHT1-163 #1-5), a deletion derivative coding for positions 1 to 171 amino acid residues (pHT1-171 #1-4), a deletion derivative coding for positions 1 to 191 amino acid residues (pHT1-191 #1-4) and a deletion derivative coding for positions 1 to 211 amino acid residues (pHT1-211 #1-4). The whole length of the amplified regions of each plasmid DNA was subjected to nucleotide sequence analysis to find its complete identity with the nucleotide sequence shown in SEQ ID NO:4.

Transfection to COS 1 cells of the thus obtained clones was carried out according to the procedure of Example 11. That is, transfection was performed with 10 µg of each of the plasmid DNA samples by the DEAE-dextran method which includes chloroquine treatment. The transfected COS1 cells were cultured for 3 days and then the culture supernatants were recovered.

The culture supernatants were extensively dialyzed against the IMDM culture medium and evaluated by the rat CFU-MK assay system. TPO activity was detected in a dose-dependent fashion in the supernatant of COS1 cells in which pHT1-211, pHT1-191, pHT1-171 or pHT1-163 each was expressed. Representative data with pHT1-211#1, pHT1-191#1 and pHT1-171#2 is shown in Fig. 13a and with pHT1-163#2 in Fig. 13b. Similar results were obtained in the M-07e assay system. The supernatant of COS1 cells, in which pHT1-211, pHT1-191, pHT1-171 or pHT1-163 each was expressed,

significantly augmented M-07e cell proliferation. Representative data with pHT1-211#1, pHT1-191#1, pHT1-171#2 or pHT1-163#2 is shown in Fig. 14.

These results showed that human TPO still retains its *in vitro* biological activity even after its carboxylterminal half beyond Ser (position 163) is deleted, and strongly suggested that the biologically active portions of human TPO localize within the amino-terminal half ending at Ser (position 163).

<Example 28>
Preparation of C-terminal deletion type human TPO and its expression and activity in COS 1 cells

In order to analyze the region necessary for the human TPO protein that exhibits its activity, the C-terminal amino acids were further deleted from the deletion derivatives obtained in Example 27 and the TPO activity expressed in COS 1 cells was examined. Using the plasmid clone pEF18S-HL34 obtained in Example 16, expression plasmids were prepared by deleting nucleotide sequences coding for C-terminal amino acid residues starting from the 163 position serine. Construction of these deletion plasmids was effected making use of PCR. Sequences of primers prepared for use in PCR are as follows:

hTPO-5: 5'-TTT GAA TTC GGC CAG CCA GAC ACC CCG GCC-3' (SEQ ID NO:61) (prepared by adding an *EcoRI* recognition sequence to the sequence of positions 1 to 21 in SEQ ID NO: 4; the same sequence shown in Example 18);

hTPO-150: 5'-TTT GCG GCC GCT CAT TAG AGG GTG GAC CCT CCT ACA AGC AT-3' (SEQ ID NO:62) (an antisense primer corresponding to the sequence of positions 514 to 537 in SEQ ID NO:4; prepared by adding two termination codons TAA and TGA and a *Not*I recognition sequence for use in the

preparation of a deletion mutant coding for positions 1 to 150 amino acid residues);

hTPO-151: 5'-TTT GCG GCC GCT CAT TAG CAG AGG GTG GAC CCT CCT ACA A-3' (SEQ ID NO:63) (an antisense primer corresponding to the sequence of positions 518 to 540 in SEQ ID NO:4; prepared by adding two termination codons TAA and TGA and a *Not*Il recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 151 amino acid residues):

hTPO-153: 5'-TTT GCG GCC GCT CAT TAC CTG ACG CAG AGG GTG GAC CC-3' (SEQ ID NO:64) (an antisense primer corresponding to the sequence of positions 526 to 546 in SEQ ID NO:4; prepared by adding two termination codons TAA and TGA and a *Not*Il recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 153 amino acid residues);

hTPO-154: 5'-TTT GCG GCC GCT CAT TAC CGC CTG ACG CAG AGG GTG GA-3' (SEQ ID NO:65) (an antisense primer corresponding to the sequence of positions 529 to 549 in SEQ ID NO:4, prepared by adding two termination codons TAA and TGA and a *Not*I recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 154 amino acid residues);

hTPO-155: 5'-TTT GCG GCC GCT CAT TAG GCC CGC CTG ACG CAG AGG GT-3' (SEQ ID NO:66) (an antisense primer corresponding to the sequence of positions 532 to 552 in SEQ ID NO:4, prepared by adding two termination codons TAA and TGA and a *Not*I recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 155 amino acid residues);

hTPO-156: 5'-TTT GCG GCC GCT CAT TAT GGG GCC CGC CTG ACG CAG AG-3' (SEQ ID NO:67) (an antisense primer corresponding to the sequence of positions 535 to 555 in SEQ ID NO:4, prepared by adding two termination codons TAA and

TGA and a *Not*1 recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 156 amino acid residues); and

hTPO-157: 5'-TTT GCG GCC GCT CAT TAG GGT GGG GCC CGC CTG ACG CA-3' (SEQ ID NO:68) (an antisense primer corresponding to the sequence of positions 538 to 558 in SEQ ID NO:4, prepared by adding two termination codons TAA and TGA and a *Not*I recognition sequence for use in the preparation of a deletion derivative coding for positions 1 to 157 amino acid residues).

PCR was carried out using 1 µg of plasmid DNA of the clone pEF18S-HL34 obtained in Example 16 as a template and 10  $\mu M$  each of the thus synthesized oligonucleotides (hTPO-5 for 5' side and hTPO-150, -151, -153, -154, -155. -156 and -157 for 3' side) as primers. Using GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTag<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), the PCR reaction was performed in 100 μl volume making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and repeating a total of 20 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 66 c for 1 minute and synthesis at 72 c for 1 minute, followed by the final incubation at 72°c for 7 minutes. A band of interest thus obtained by each PCR was digested with restriction enzymes EcoRI and NotI and the resulting digest was subjected to 1% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a main DNA fragment having a size expected by each PCR. The thus isolated DNA fragment was purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and then subcloned into the expression vector pEF18S which has been treated in advance with the same restriction enzymes. In this case, Competent-High E. coli DH5 manufactured by TOYOBO was used as the host strain. From the resulting transformants which originated from each PCR experiment, 3 to 5 clones

containing the insert of expected size were selected to prepare plasmid DNA samples basically in accordance with the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). By a series of such operations, plasmid DNA samples were obtained from a deletion derivative coding for positions 1 to 150 amino acid residues (pHT1-150 # 21, 22 and 25), a deletion derivative coding for positions 1 to 151 amino acid residues (pHT1-151 # 16, 17 and 18), a deletion derivative coding for positions 1 to 153 amino acid residues (pHT1-153 # 1 to 5), a deletion derivative coding for positions 1 to 154 amino acid residues (pHT1-154 # 1 to 5), a deletion derivative coding for positions 1 to 155 amino acid residues (pHT1-155 # 1 to 5), a deletion derivative coding for positions 1 to 156 amino acid residues (pHT1-156 # 1 to 5) and a deletion derivative coding for positions 1 to 157 amino acid residues (pHT1-157 # 1 to 5).

Of these purified plasmid DNA samples, the sequence of pHT1-150 # 21, 22 and 25 and pHT1-151 # 16, 17 and 18 were checked by 373A DNA Sequencer manufactured by Applied Biosystems making use of Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (Applied Biosystems), thereby confirming expected TPO cDNA sequences with no substitutions over the entire nucleotide sequences.

Transfection of COS 1 cells with each of the thus obtained clones was carried out in accordance with the procedure of Example 11. That is, transfection was performed with 10 µg of each of the plasmid DNA samples by the DEAE-dextran method which includes chloroquine treatment, and the culture supernatant was recovered after 3 days of the culture. The thus obtained culture supernatants were dialyzed against the IMDM culture medium described in the foregoing and evaluated by the M-07e assay system. TPO activities were detected in the culture supernatant of COS 1 cells transfected with respective clones coding for C-terminal deletion

derivatives, consisting of 1 to 151 position amino acids, 1 to 153 position amino acids, 1 to 154 position amino acids, 1 to 155 position amino acids, 1 to 156 position amino acids or 1 to 157 position amino acids. All of these derivatives contain a cysteine residue at position 151. However, no TPO activities were detected in the culture supernatant of COS 1 cells transfected with the clone coding for a C-terminal side deletion derivative consisting of 1 to 150 position amino acids whose cysteine residue at the 151 position was also deleted.

<Example 29>
Preparation of N-terminal deletion type human TPO and its expression and activity in COS 1 cells

In order to analyze the region necessary for the TPO protein that exhibits its biological activity, the N-terminal side amino acids of the deletion derivatives obtained in Example 28 were further deleted and expression of TPO activity of the thus prepared deletion derivativess was examined. Using the plasmid clone pEF18S-HL34 obtained in Example 16 and the plasmid clone pHT1-163 obtained in Example 27, expression plasmids were prepared by deleting nucleotide sequences coding for N-terminal amino acid residues after the signal sequence. Construction of these deletion plasmids were effected making use of PCR. Sequences of primers prepared for use in PCR are as follows:

hTPO-5: 5'-TTT GAA TTC GGC CAG CCA GAC ACC CCG GCC-3' (SEQ ID NO:69) (prepared by adding an *EcoRI* recognition sequence to the sequence of positions 1 to 21 in SEQ ID NO:4; the same sequence shown in Example 18);

hTPO3: 5'-TTT GCG GCC GCT CAT TAT TCG TGT ATC CTG TTC AGG TAT CC-3' (SEQ ID NO:70) (an antisense primer corresponding to the sequence of positions 757 to 780 in SEQ ID NO:4; prepared by adding two termination codons TAA and

TGA and a *Not*1 recognition sequence; the same sequence synthesized for use in the preparation of a deletion derivative coding for positions 1 to 231 amino acid residues);

hTPO-S: 5'-TTT GCG GCC GCT CAT TAG CTG GGG ACA GCT GTG GTG GGT-3' (SEQ ID NO:71) (an antisense primer corresponding to the sequence of positions 555 to 576 in SEQ ID NO:4; prepared for use in the preparation of a deletion derivative coding for positions 1 to 163 amino acid residues);

hTPO-13: 5'-AGT AAA CTG CTT CGT GAC TCC CAT GTC CTT CAC AGC AGA CTG AGC CAG TG-3' (SEQ ID NO:72) (positions 124 to 173 in SEQ ID NO:4; prepared for use in the preparation of a derivative in which amino acid residues of positions 1 to 12 are deleted);

hTPO-13R: 5'-CAT GGG AGT CAC GAA GCA GTT TAC TGG ACA GCG TTA GCC TTG CAG TTA G-3' (SEQ ID NO:73) (an antisense primer corresponding to the sequence of positions 64 to 87 and 124 to 148 in SEQ ID NO: 4, prepared for use in the preparation of a derivative in which amino acid residues of positions 1 to 12 are deleted);

hTPO-7: 5'-TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC CTT C-3' (SEQ ID NO:74) (positions 106 to 154 in SEQ ID NO:4, prepared for use in the preparation of a derivative in which amino acid residues of positions 1 to 6 are deleted):

hTPO-7R: 5'-TTT ACT GAG GAC TCG GAG GTC ACA GGA CAG CGT TAG CCT TGC AGT TAG-3' (SEQ ID NO:75) (an antisense primer corresponding to the sequence of positions 64 to 87 and 106 to 129 in SEQ ID NO:4, prepared for use in the preparation of a derivative in which amino acid residues of positions 1 to 6 are deleted);

hTPO-8: 5'-GAC CTC CGA GTC CTC AGT AAA CTG-CTT CGT GAC TCC CAT GTC CTT CAC A-3' (SEQ ID NO:76) (positions 109 to 157 in SEQ ID NO:4, prepared for use in the preparation of a derivative in which amino acid residues of positions 1 to 7 are deleted); and

hTPO-8R: 5'-CAG TTT ACT GAG GAC TCG GAG GTC GGA CAG CGT TAG CCT TGC AGT TAG-3' (SEQ ID NO:77) (an antisense primer corresponding to the sequence of positions 64 to 87 and 109 to 132 in SEQ ID NO:4, prepared for use in the preparation of a derivative in which amino acid residues of positions 1 to 7 are deleted).

(1) Preparation of a derivative (pHT13-231) in which amino acid residues of positions 1 to 12 are deleted

PCR was carried out using 1.4 µg of plasmid DNA of the clone pEF18S-HL34 obtained in Example 18 as a template and 5  $\mu M$  each of the thus synthesized oligonucleotides (hTPO-13 and hTPO3 in one combination and hTPO-5 and hTPO-13R in another combination) as primers. Using GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), the PCR reaction was performed in 100 μl volume making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and, after 5 minutes of denaturation at 95 c, repeating a total of 30 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 65°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes. Each of the PCR products was subjected to 1.2% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a main DNA fragment having an expected size which was subsequently purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and dissolved in 15 µl of TE buffer. Thereafter, second PCR was carried out using 1 µl portion of each of the thus prepared solution as a template.

The second PCR was carried out using 5 µM each of the synthesized primers (hTPO-5 and hTPO3). Using GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), the PCR reaction was

performed in 100 µl volume making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and, after 5 minutes of denaturation at 95°c, repeating a total of 30 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 60°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 Each of the PCR products was subjected to 1.2% minutes. gel (manufactured by FMC BioProducts) agarose electrophoresis to isolate a main DNA fragment having an expected size which was subsequently purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and dissolved in 15 µl of TE buffer. After digestion with restriction enzymes EcoRI and NotI, the resulting solution was subjected to extraction with the same volume of phenol/chloroform and subsequent ethanol precipitation. After centrifugation, the resulting precipitate was dissolved in 15 μl of TE buffer and then sub-cloned into the expression vector pEF18S which has been treated in advance with the same restriction enzymes. In this case, Competent-High E. coli DH5 manufactured by TOYOBO was used as the host strain. From the resulting transformants, 45 clones containing the insert of expected size were selected to prepare plasmid DNA samples basically in accordance with the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). In this way, it was able to obtain plasmid DNA of a deletion derivative (pHT13-231) coding for a protein molecule in which amino acids residues of positions 1 to 12 have been deleted from the original amino acid residues of positions 1 to 231. When these 45 clones were subjected to PCR using primers hTPO-5 and hTPO3, inserts having approximately expected sizes were confirmed in 8 clones. Of these, the sequence of 3 clones were checked by 373A DNA Sequencer manufactured by Applied Biosystems making use of Tag Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (Applied

Biosystems), thereby obtaining a clone pHT13-231 #3 having a TPO cDNA sequence as designed and a deletion as expected with no substitutions and the like over the entire nucleotide sequence.

(2) Preparation of a derivative (pHT7-163) in which amino acid residues of positions 1 to 6 are deleted

In this case, the 163 position amino acid was used as the C-terminal for the preparation of deletion derivativess because, though the 231 position amino acid was designed as the C-terminal of TPO protein for the preparation of deletion derivativess in the above step (1), it was found that TPO activity could be expressed even when the C-terminal amino acids were further deleted. On the basis of the same reason, the 163 position amino acid was used as the C-terminal also in the subsequent step (3). PCR was carried out using 1.4  $\mu g$  of plasmid DNA of the clone pHT1-163 obtained in Example 27 as a template and 5  $\mu\text{M}$  each of the synthesized oligonucleotides (hTPO-7 and hTPO-S in one combination and hTPO-5 and hTPO-7R in another combination) as primers. Using GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), the PCR reaction was performed in 100 μl volume making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and, after 5 minutes of denaturation at 95°c, repeating a total of 30 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 65°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes. Each of the PCR products was subjected to 1.2% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a main DNA fragment having an expected size which was subsequently purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and dissolved in 15 µl of TE buffer.

Thereafter, second PCR was carried out using 1  $\mu$ l portion of each of the thus prepared solution as a template.

The second PCR was carried out using 5 µM each of the synthesized primers (hTPO-5 and hTPO-S). GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), the PCR reaction was performed in 100 μl volume making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and, after 5 minutes of denaturation at 95°c, repeating a total of 30 cycles, each cycle consisting of denaturation at 95 c for 1 minute, annealing at 60°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes. Each of the PCR products was subjected to 1.2% **FMC** BioProducts) (manufactured by agarose ael electrophoresis to isolate a main DNA fragment having an expected size which was subsequently purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and dissolved in 15  $\mu$ l of TE buffer. After digestion with restriction enzymes EcoRI and NotI, the resulting solution was subjected to extraction with the same phenol/chloroform and subsequent ethanol precipitation. centrifugation, the resulting precipitate was dissolved in 15 μl of TE buffer and then sub-cloned into the expression vector pEF18S which has been treated in advance with the same restriction enzymes. In this case, Competent-High E. coli DH5 manufactured by TOYOBO was used as the host strain. From the resulting transformants, 30 clones containing the insert of expected size were selected to prepare plasmid DNA samples basically in accordance with the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). In this way, it was able to obtain plasmid DNA of a deletion derivative (pHT7-163) coding for a protein molecule in which amino acids residues of positions 1 to 6 have been deleted from the original amino acid residues of positions 1 to 163 in SEQ ID NO: 4. When these clones were subjected to PCR using primers hTPO-5 and hTPO-S, inserts having approximately expected sizes were confirmed in all clones. Of these, the sequences of 3 clones were checked by 373A DNA Sequencer manufactured by Applied Biosystems making use of Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (Applied Biosystems), thereby obtaining 2 clones, pHT7-163 #4 and #29, each having a TPO cDNA sequence as designed and a deletion as expected with no substitutions and the like over the entire nucleotide sequence.

## (3) Preparation of a derivative (pHT8-163) in which amino acid residues of positions 1 to 7 are deleted

A derivative (pHT8-163) having deletion of amino acid residues of positions 1 to 7 was prepared basically in the same manner as the above case for the preparation of a derivative (pHT7-163) having deletion of amino acid residues of positions 1 to 6. PCR was carried out using 1.4 μg of plasmid DNA of the clone pHT1-163 obtained in Example 27 as a template and 5 μM each of the synthesized oligonucleotides (hTPO-8 and hTPO-S in one combination and hTPO-5 and hTPO-8R in another combination) as primers. Using GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTag<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), the PCR reaction was performed in 100 µl volume making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and, after 5 minutes of denaturation at 95°c, repeating a total of 30 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 65°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 minutes. Each of the PCR products was subjected to 1.2% agarose gel (manufactured by FMC BioProducts) electrophoresis to isolate a main DNA fragment having an expected size which was subsequently purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and dissolved in 15  $\mu$ l of TE buffer. Thereafter, second PCR was carried out using 1  $\mu$ l portion of each of the thus prepared solutions as a template.

The second PCR was carried out using 5 µM each of the synthesized primers (hTPO-5 and hTPO-S). GeneAmp<sup>TM</sup> PCR Reagent Kit with AmpliTaq<sup>TM</sup> DNA Polymerase (manufactured by Takara Shuzo), the PCR reaction was performed in 100  $\mu l$  volume making use of GeneAmp<sup>TM</sup> PCR System 9600 (manufactured by PERKIN-ELMER) and, after 5 minutes of denaturation at 95°C, repeating a total of 30 cycles, each cycle consisting of denaturation at 95°c for 1 minute, annealing at 60°c for 1 minute and synthesis at 72°c for 1 minute, followed by the final incubation at 72°c for 7 Each of the PCR products was subjected to 1.2% minutes. gel (manufactured by FMC BioProducts) electrophoresis to isolate a main DNA fragment having an expected size which was subsequently purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad) and dissolved in 15 µl of TE buffer. After digestion with restriction enzymes EcoRl and Notl, the resulting solution was subjected to extraction with the same volume of phenol/chloroform and subsequent ethanol precipitation. After centrifugation, the resulting precipitate was dissolved in 15 ul of TE buffer and then sub-cloned into the expression vector pEF18S which has been treated in advance with the same restriction enzymes. In this case, Competent-High E. coli DH5 manufactured by TOYOBO was used as the host strain. From the resulting transformants, 30 clones containing the insert of expected size were selected to prepare plasmid DNA samples basically in accordance with the procedure described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989). In this way, it was able to obtain plasmid DNA of a deletion derivative (pHT8-163) coding for a protein molecule in which amino acids residues of positions 1

to 7 have been deleted from the original amino acid residues of positions 1 to 163 in SEQ ID NO:4. When these clones were subjected to PCR using primers hTPO-5 and hTPO-S, inserts having approximately expected sizes were confirmed in all clones. Of these, the sequence of 3 clones were checked by 373A DNA Sequencer manufactured by Applied Biosystems making use of Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (Applied Biosystems), thereby obtaining 2 clones, pHT8-163 #33 and #48, each having a TPO cDNA sequence as designed and a deletion as expected with no substitutions and the like over the entire nucleotide sequence.

# (4) Expression of deletion derivative in COS 1 cells and confirmation of TPO activity

Transfection of COS 1 cells with each of the thus obtained deletion clones was carried out according to the procedure of Example 11. That is, transfection was performed with 10 µg of each of the plasmid DNA samples by the DEAE-dextran method which includes chloroquine treatment, and the culture supernatants were recovered after 3 days of culture. The thus obtained culture supernatant was thoroughly dialyzed against the IMDM culture medium described in the foregoing and evaluated by the M-07e assay system.

As a result, weak, but significant, TPO activity was detected in the culture supernatant of COS 1 cells transfected with a clone coding for the deletion derivative consisting of 7 to 163 position amino acids. However, no TPO activity was detected in the culture supernatant of COS 1 cells transfected with a clone coding for the deletion derivative consisting of 8 to 163 position amino acids or of 13 to 231 position amino acids.

<Example 30>

Preparation of complete length of human TPO cDNA plasmid (pHTP1)

An expression vector having all amino acid coding regions of human TPO cDNA presumed in SEQ ID NO:6 was constructed for the expression in mammalian cells.

PCR was carried out in the following manner in order to prepare a DNA fragment which covers all human TPO cDNA coding regions.

Nucleotide sequences of the used primers are as follows:

hTPO-I: 5'-TTG TGA CCT CCG AGT CCT CAG-3' (SEQ ID NO:78) (positions 105 to 125 in SEQ ID NO:6);

SA: 5'-CAG GTA TCC GGG GAT TTG GTC-3' (SEQ ID NO:79) (an antisense primer corresponding to the sequence of positions 745 to 765 in SEQ ID NO:6);

hTPO-P: 5'-TGC GTT TCC TGA TGC TTG TAG-3' (SEQ ID NO:80) (positions 503 to 523 in SEQ ID NO:6); and

hTPO-KO: 5'-GAG AGA GCG GCC GCT TAC CCT TCC TGA GAC. AGA TT-3' (SEQ ID NO:81) (a sequence prepared by adding a restriction enzyme *Not*I recognition sequence and a GAGAGA sequence (SEQ ID NO:82) to an antisense sequence corresponding to the sequence of positions 1066 to 1086 in SEQ ID NO:6).

A first PCR was carried out using 300 ng of the clone pEF18S-HL34 obtained in Example 16 as a template. Using 0.5 μM each of the primers hTPO-I and SA and 1 unit of Vent R<sup>TM</sup> DNA polymerase (manufactured by New England BioLabs), the PCR (repetition of a total of 30 cycles, each cycle consisting of incubations at 96 c for 1 minute, at 62 c for 1 minute and at 72 c for 1 minute, followed by the final incubation at 72 c for 7 minutes) was carried out. Composition of the reaction solution in final concentration is as follows: 10 mM KCI, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCI (pH 8.8), 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100 and 200 μM dNTP mix.

One microgram portion of commercially available poly(A)+ RNA preparation derived from human normal liver (manufactured by Clontech) was heated at 70°c for 10 minutes, rapidly cooled on ice and then mixed with 10 mM of DTT, 500  $\mu M$  of dNTP mix, 25 ng of random primer (manufactured by Takara Shuzo), 10 units of RNase Inhibitor (manufactured by Boehringer-Mannheim) and 200 units of SuperScript TM II RNase H (manufactured by LIFE TECHNOLOGIES), followed by 1 hour of incubation at 37°c to perform synthesis of cDNA. Thereafter, second PCR (repetition of a total of 30 cycles, each cycle consisting of incubations at 96 °C for 1 minute, at 58 °C for 1 minute and at 72 °C for 1 minute) was carried out using 1/20 volume of the reaction solution of synthesized cDNA as a template and 2.5 μM each of the primers hTPO-P and hTPO-KO and 2.5 units of AmpliTaqTM DNA polymerase (manufactured by Takara Shuzo).

Each of the resulting solutions of the first and second PCR was subjected to 1% agarose gel electrophoresis to isolate respective main DNA fragments of expected size which was subsequently purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad). Thereafter, third PCR was carried out using 1/20 volume of each of the thus prepared solutions as a template. This reaction (heating at 96 °C for 2 minutes, followed by the repetition of a total of 3 cycles, each cycle consisting of incubations at 96°c for 2 minute and at 72°c for 2 minute, and subsequent incubation at 72°c for 7 minutes) was carried out using 1 unit of Vent RTM DNA polymerase (manufactured by New England BioLabs). The resulting reaction solution was mixed with 1 µM each of hTPO-I and hTPO-KO, heated at 96 c for 2 minutes, subjected to 25 cycles of reaction, each cycle consisting of incubations at 96 c for 1 minute, at 62 c for 1 minute and at 72 c for 1 minute, and then incubated at 72°c for 7 minutes. The resulting reaction solution was extracted with the same

volume of water saturated phenol-chloroform and then with the same volume of chloroform, and the extract was subjected to ethanol precipitation (2.5 volumes of ethanol in the presence of 0.3 M sodium acetate and 0.5 µl of glycogen manufactured by Boehringer-Mannheim) to recover DNA. The thus recovered DNA was digested with restriction enzymes Bam HI and Not1 and subjected to 1% agarose gel electrophoresis, and a main band having an expected size thus isolated was purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad), ligated with pBluescript II SK+ vector (manufactured by Stratagene) which had been digested in advance with the restriction enzymes BamHI and NotI and into Competent-High E. coli DH5 transformed (manufactured by TOYOBO). From the resulting colonies, 4 clones were selected to prepare plasmid DNA samples. sequence of purified plasmid DNA samples were checked by 373A DNA Sequencer manufactured by Applied Biosystems making use of Tag Dye Deoxy<sup>TM</sup> Terminater Cycle Sequening Kit (Applied Biosystems), thereby obtaining a clone, pBLTP, having a TPO cDNA sequence as designed with no substitution in nucleotide sequence within the region between BamHI and Notl.

The clone pBLTP was digested with restriction enzymes *EcoRI* and *BamHI* and subjected to 1% agarose gel electrophoresis, and a band of high molecular weight thus isolated was purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad). In the same manner, pEF18S-HL34 was treated with the restriction enzymes to purify a DNA fragment of about 450 bp. Each of the thus obtained DNA samples was subjected to ligation and transformed into Competent-High *E. coli* DH5 (manufactured by TOYOBO). Plasmid DNA samples were prepared from the resulting colonies to obtain a clone, pBLTEN, containing human TPO cDNA insert. The thus obtained pBLTEN was digested with restriction enzymes *EcoRI* and *NotI* and subjected to 1%

agarose gel electrophoresis, and a DNA fragment of about 1,200 bp thus isolated was purified using Prep-A-Gene DNA Purification Kit (manufactured by Bio-Rad), ligated with the expression vector pEF18S which has been digested in advance with the same restriction enzymes and then transformed into Competent-High *E. coli* DH5 (manufactured by TOYOBO). Plasmid DNA samples were prepared from the resulting colonies to obtain the clone of interest, pHTP1, which contains entire human TPO cDNA coding region. Plasmid DNA was prepared from this clone in a large quantity and used in the following experiments. Preparation of plasmid DNA was carried out basically in accordance with the procedure described in *Molecular Cloning* (Sambrook *et al.*, Cold Spring Harbor Laboratory Press, 1989).

<Example 31>

Construction of mammalian expression plasmid. pDEF202hTPO-P1. for the expression of human TPO in Chinese hamster ovary (CHO) cells.

One microgram of a plasmid pMG1 containing mouse dihydrofolate reductase (DHFR) minigene was digested with restriction enzymes, EcoRI and BamHI, and the subjected to agarose gel electrophoresis to isolate a fragment (about 2.5 kb) containing mouse DHFR minigene. The isolated DNA fragment was dissolved in 25 ml of a reaction solution composed of 50 mM Tris-HCl (pH 7.5), 7 mM MgCl<sub>2</sub>, 1 mM 2mercaptoethanol and 0.2 mM dNTP mixture, mixed with 2 units of Klenow fragment and then incubated at room temperature for 30 miniutes to form blunt ends at both termini of the DNA After phenol/chloroform treatment and ethanol precipitation, the resulting fragment was dissolved in 10 ml of a TE solution (10 mM Tris-HCl (pH 8.0)/1 mM EDTA). The mammalian expression vector pEF18S was digested with a restriction enzyme Smal, dephosphorylated with alkaline

phosphatase (manufactured by Takara Shuzo) and then ligated with a DNA fragment containing mouse DHFR minigene by using T4 DNA ligase (manufactured by Takara Shuzo) to obtain an expression vector pDEF202.

Next, the constructed expression vector pDEF202 was digested with restriction enzymes, EcoRI and Spel, and then subjected to agarose gele electrophoresis to isolate a larger DNA fragment. Human TPO cDNA which has been obtained by digesting the plasmid pHTP1 containing human TPO cDNA (clone p1) with restriction enzymes, EcoRI and Spel, was ligated with this linearized pDEF202 vector by using T4 DNA ligase (manufactured by Takara Shuzo) to obtain a plasmid pDEF202-hTPO-P1 for expression of human TPO cDNA. constructed plasmid contains SV40 replication origin, human 1-α-promoter, and elongation factor polyadenylation signal for transcription of human TPO cDNA, mouse DHFR minigene, and pUC18-replication origin and βlactamase gene (Amp<sup>r</sup>).

#### <Example 32>

#### Expression of human TPO in CHO cells

CHO cells (a DHFR- strain; Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, vol.77, p4216, 1980) were maintained in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM(-), supplemented with hypoxanthine and thymidine) containing 10% fetal calf serum (FCS) by using a 6 cm diameter tissue culture plate (by Falcon). The transformation of CHO cells with a pDEF202-hTPO-P1 plasmid was carried out by a calcium phosphate method (CellPhect, manufactured by Pharmacia) as described below. Ten micrograms of the plasmid pDEF202-hTPO-P1 prepared in Example 31 was mixed with 120 ml of buffer A and 120 ml of H 2O, and the resulting mixture was incubatedat room temperature for 10 minutes. This solution was further mixed with 120 ml of buffer B and allowed to stand at room

This DNA mixture temperature for additional 30 minutes. solution was then added into the culture and incubated for 6 hours in a CO2 incubator. After 6 hours, the culture was washed twice with serum-free  $\alpha$ -MEM(-), treated with  $\alpha$ -MEM(-) containing 10% dimethyl sulfoxide for 2 minutes, and then incubated for 2 days in non-selection medium ( $\alpha\text{-MEM}(-)$ supplemented with hypoxanthine and thymidine) containing 10 % dialyzed FCS. After 2 days culture, the cells were selected in a selection medium ( $\alpha$ -MEM(-)) containing 10% dialyzed FCS. For selection of transformed cells with DHFR-positive phenotype, the cells were treated with a trypsin/EDTA solution, and resuspended with a selection medium. The cells in 6 cm tissue culture plate were split into five 10 cm tissue culture plates or twelve 24-well tissue culture plates, and then cultured in the selection medium. The culture medium was exchanged at the intervals of two days. The human TPO activity in the cultured medium of CHO cells with DHFRpositive phenotype was measured by CFU-MK assay, M-O7e assay or Ba/F3 assay. The cells secreting human TPO into culture medium were further selected in the selection medium supplemented with 25 nM methotrexate in order to isolate the cell clone producing a higher amount of human TPO.

In this instance, transfection of CHO cells may also be effected by carrying out co-transfection of CHO cells with pHTP1 and pMG1 plasmids.

CHO strain (CHO-DUKXB11) transfected with the plasmid pDEF202-hTPO-P1 has been deposited by the present applicant on January 31, 1995, under the accession No. FERM BP-4988, at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

<Example 33>
Construction of a recombinant vector,

### BMCGSneo-hTPO-P1. for use in X 63.6.5.3. cells

One microgram of a mammalian expression vector pBMCGSneo was digested with restriction enzymes, Xhol and Notl, and the subjected to agarose gel electrophoresis to isolate the vector DNA portion. This DNA fragment and human TPO cDNA (P1 clone) which had been obtained by digesting the plasmid pBLTEN containing human TPO cDNA with the restriction enzymes Xhol and Notl were ligated using T4 DNA ligase to obtain the expression vector of interest, pBMCGSneo-This expression plasmid contains cytomegalovirus early promoter, parts of rabbit  $\beta$  globin gene-derived intron and polyadenylated region for transcription of human TPO cDNA, human β globin gene, 69% of bovine papilloma virus 1 gene, thymidine kinase promoter and polyadenyl region. phosphotransferase I gene (neomycin resistant gene) and pBR 322 replication origin and β-lactamase gene (Amp<sup>r</sup>), and human TPO cDNA was ligated at a downstream site of the cytomegalovirus promoter.

#### <Example 34>

#### Expression in X 63.6.5.3.cells

X 63.6.5.3.cells were cultured in Dulbecco's minimal essential (DME) medium containing 10% fetal bovine serum. The transfection of X63.6.5.3 cells with BMCGSneo hTPO plasmid was carried out by electroporation method as described below.

20 micrograms of the plasmid BMCGSneo-hTPO-P1 prepared in Example 33 were added to 750  $\mu$ l of the DME medium containing 10<sup>7</sup> cells, and the mixture was put into an electroporation cuvette having a gap of 4 mm and allowed to stand for 10 minutes at 4°c. The cuvette was then set into an electroporation apparatus (BTX 600, manufactured by BTX) to carry out gene transfer under conditions of 380 V, 25 mF and 24 angstroms. After the gene transfer, the cuvette was again

allowed to stand for 15 minutes at 4°c, and the cells were then washed once with DEM containing 10% fetal bovine serum. The transfected cells were suspended in 50 ml of DEM containing 10% fetal bovine serum, dispensed into wells of five 96 well tissue culture plate and then cultured for 2 days in a CO<sub>2</sub> incubator. After the 2 days culture, the culture medium was exchanged with DEM containing 10% fetal bovine serum and 1 mg/ml of G418 (manufactured by GIBSCO), and the medium exchange was carried out every 3 days thereafter. The human TPO activity in the culture medium of transformants was mesured by CFU-MK, M-07e or Ba/F3 assay. The transformants secreting human TPO into culture medium were cloned twice by a limiting dilution to establish a human TPO producing cell lines.

#### <Example 35>

### Large scale expression of human TPO in COS 1 cells

Transfection of COS 1 cells was carried out according to the DEAE-dextran method which includes chloroquine treatment as described in Example 11. COS 1 cells (ATCC CRL1650) were cultured in IMDM containing 10% (v/v) FCS using a collagen-coated 175 cm<sup>2</sup> culture flask at 37°c in a 5% carbon dioxide incubator until the cells became about 100% confluent. In order to effect collagen coating of the culture flask, 25 ml of a collagen solution (Cellmatrix type I-C, manufactured by Iwaki) whose concentration has been adjusted to 0.3 mg/ml with 1 mM HCl was added to each 175 cm<sup>2</sup> culture flask, the collagen solution was recovered after 1 hour of standing at room temperature and then the thus treated flask was washed once with 20 to 50 ml of PBS. Transfection was effected by mixing 20 ml of IMDM solution containing 250 μg/ml of DEAE-dextran (manufactured by Pharmacia), 60 μM of chloroquine (manufactured by Sigma) and 10% (v/v) of Nu-Serum (manufactured by Collaborative) with the plasmid

pHTP1 (40 μg) which has been dissolved in 500 μl of HBS, adding the resulting mixture to the COS 1 cells described above contained in one 175 cm<sup>2</sup> culture flask, which have been washed once with IMDM just before the transfection, and then culturing the cells for 3 hours at 37°c in a 5% carbon dioxide incubator. Thereafter, the culture supernatant was removed by suction, and the cells were washed once with IMDM, mixed with 50 ml of a serum-free medium and then cultured for 5 days at 37°c in the 5% carbon dioxide incubator to recover the culture supernatant. In one operation, 100 to 260 culture flasks of 175 cm<sup>2</sup> were used and 5 to 13 liters of the culture supernatant was recovered. The serum-free medium was prepared by supplementing IMDM with 5 μg/ml of insulin (manufactured by Sigma), μg/ml of transferrin 5 (manufactured by Sigma), 10 μM of monoethanolamine (manufactured by Wako Pure Chemical Industries), 25 nM of sodium selenite (manufactured by Sigma) and 200 μg/ml of BSA (fatty acid-free high purity bovine albumin, manufactured by Nichirei).

<Example 36>

Purification of human full-length TPO (derived from expression plasmid pHTP1. P1 clone) expressed in COS 1 cells and its biological activity

The following describes examples of the activity and purification of human full-length TPO (derived from the expression plasmid pHTP1) expressed in COS 1 cells. A partially purified product was firstly prepared in order to examine platelet increasing activity and then the purification was carried out to obtain a high purity product. The M-07e assay system was mainly used for the measurement of TPO activity in the following preparation steps. TPO activity was similarly found in the rat CFU-MK assay system. In carrying

out these assays, human serum albumin (HSA) was added to each sample to a final concentration of 0.02 to 0.05%.

Firstly, COS 1 cells transfected with the plasmid pHTP1 according to the method of Ohashi and Sudo (Hideya Ohashi and Tadashi Sudo. Biosci. Biotech. Biochem., 58 (4), 758 - 759, 1994) were cultured for 5 days at 37°c in a 5% CO2 using a serum-free IMDM culture medium containing, 0.2 g of BSA, 5 mg of bovine insulin, 5 mg of human transferrin, 0.02 mM of monoethanolamine and 25 nM of sodium selenite, in 1000ml, thereby obtaining about 7 liters of serum-free culture supernatant. To the serum-free culture supernatant was added proteolytic enzyme inhibitors p-APMSF and Pefabloc SC (4-(2aminoethyl)-benzenesulfonyl fluoride hydrochloride, manufactured by Merk, catalog No. 24839) to a final concentration of about 1 mM, followed by filtration using a 0.2 μm filter to recover the supernatant. This was concentrated by a factor of about 10 using an ultrafiltration unit (Omega Ultrasette, nominal molecular weight cutoff of 8,000, manufactured by Filtron; or PLGC Pellicon Cassette, nominal molecular weight cutoff of 10,000, manufactured by Millipore), 723 ml of the thus concentrate (protein concentration, 3.38 mg/ml; total protein, 2,445 mg; relative activity, 43,000; and total activity, 105,100,000), was mixed with 1.6 moles per 1,000 ml of ammonium sulfate (288 g in total) to obtain 804 ml of the solution containing 1.5 M in final concentration of ammonium sulfate, and then the thus obtained solution was applied at a flow rate of 10 ml/min to Macro-Prep Methyl HIC column (5 cm in diameter and 9 cm in bed height, manufactured by Bio-Rad, catalog No. 156-0080) which has been equilibrated in advance with 20 mM sodium citrate buffer (pH 5.2) containing 1.5 M ammonium sulfate. After the sample loading, elution was carried out with 20 mM sodium citrate buffer (pH 5.2) containing 1.25 M ammonium sulfate to obtain passed-through fraction F1 (2,384 ml; protein

concentration, 0.864 mg/ml; total protein, 2,061 mg; and relative activity, 6,000).

Next, the eluting solution was changed to 20 mM Na citrate, pH 5.8, to collect fraction F2 (1,092 ml; protein concentration, 0.776 mg/ml; total protein, 847 mg; and relative activity, 150,000).

The thus obtained Macro-Prep Methyl HIC column fraction F2 (1,081 ml) was applied at a flow rate of 10 ml/min to SP Sepharose Fast Flow column (3 cm in diameter and 10 cm in bed height, manufactured by Pharmacia Biotech, catalog No. 17-0729-01) which has been equilibrated in advance with 20 mM sodium citrate buffer (pH 5.8). After the sample loading, elution was carried out with 20 mM sodium citrate buffer (pH 5.6) containing 110 mM NaCl to obtain fraction F1 (2,262 ml; protein concentration, 0.270 mg/ml; total protein, 610 mg; and relative activity, 30,000). Next, the eluting solution was changed to 20 mM sodium citrate buffer (pH 5.4) containing 400 mM NaCl to collect fraction F2 (856 ml; protein concentration, 0.189 mg/ml; total protein, 162 mg; and relative activity, 300,000). Next, the eluting solution was changed to 20 mM sodium citrate buffer (pH 5.2) containing 1,000 mM NaCl to collect eluted fraction F3 (370 ml; protein concentration, 0.034 mg/ml; total protein, 12.6 mg; and relative activity, 150,000).

The main TPO active fraction F2 (845 ml) of the SP Sepharose Fast Flow column step was mixed with about 10% in final concentration of 1-propanol and applied at a flow rate of 3 ml/min to LA-WGA column (2 cm in diameter and 14 cm in bed height, manufactured by Honen, catalog No. WG-007) which has been equilibrated in advance with 20 mM sodium citrate buffer (pH 5.4) containing 400 mM NaCl. After the sample loading, elution was carried out using a mixture of 20 mM sodium citrate buffer (pH 5.4) containing 400 mM NaCl with 1-propanol (9:1) to obtain fraction F1 (64.4 ml; protein

concentration, 0.0178 mg/ml; total protein, 1.15 mg; and relative activity, 17,220). Next, the eluting solution was changed to 20 mM sodium citrate buffer (pH 6.1) containing 0.4 M GlcNAc and 10% 1-propanol to collect eluted fraction F2 (45 ml; protein concentration, 0.0104 mg/ml; total protein, 0.470 mg; and relative activity, 675,000).

The main TPO active fraction F2 (340 ml) of the LA-WGA column operation was mixed with about 0.005% in final concentration of TFA and subjected to YMC-Pack CN-AP (6 mm in diameter and 250 mm in bed height, manufactured by YMC, (catalog No. AP-513) column chromatography. That is, using 0.1% TFA as a developing solvent A and 1-propanol containing 0.05% TFA as a developing solvent B, the sample was applied at a flow rate of 0.6 ml/min to the YMC-Pack CN-AP column which has been equilibrated in advance with 15% B. After the sample loading, the propanol concentration was increased from 15% B to 25% B, and the elution was carried out with a linear gradient of from 25% B to 50% B for 65 minutes while collecting the eluates in 1.5 ml (2.5 min) portions in polypropylene tubes.

A 0.5  $\mu$ I portion (1/3,000 fraction) of the eluate in each tube was mixed with HSA and concentrated by ultrafiltration to obtain 0.25 ml of IMDM assay culture solution containing 0.05% HSA for use in the identification of TPO active fractions by the assay. As the result, strong TPO activity (relative activity of about 630,000 to 4,800,000) was found in tube Nos. 24 to 30 (within the range of 35.0 and 42.0% as propanol concentration) which were pooled as TPO active fraction FA (13.5 ml).

The fraction FA obtained by the YMC-Pack CN-AP column step was subjected to Capcell Pak Cl 300A (4.6 mm in diameter and 150 mm in bed height, manufactured by Shiseido, catalog No. Cl TYPE:SG300A) column chromatography using 0.1% TFA as a developing solvent A and 1-propanol containing 0.05%

TFA as a developing solvent B. A portion (8.9 ml) of the fraction FA obtained by the YMC-Pack CN-AP column operation was mixed with 0.3 ml of glycerol, concentrated by centrifugation evaporation and then mixed with about 2.5 ml of 10% B, and the resulting solution was subsequently applied at a flow rate of 0.4 ml/min to the Capcell Pak CI 300A column which has been equilibrated in advance with 20% B. After the sample loading, the elution was carried out firstly with 20% B for 5 minutes and then with a linear gradient of from 20% B to 40% B for 50 minutes while collecting the eluates in 1 ml (2.5 min) portions in polypropylene tubes.

A 1  $\mu$ l portion (1/1,000 fraction) of the eluate in each tube was mixed with HSA and concentrated by ultrafiltration to obtain 0.25 ml of IMDM assay culture solution containing 0.05% HSA for use in the identification of TPO active fractions by the assay. As the result, strong TPO activity (relative activity of about 3,000,000 to 22,500,000) was found in tube Nos. 20 to 23 (within the range of 28.5 and 32.5% as propanol concentration) which were pooled as a high activity fraction.

#### <Example 37>

## Molecular weight measurement of human TPO expressed in COS 1 cells

It was assumed that molecular weight of the human complete length TPO (derived from the expression plasmid pHTP1, F1 clone) expressed in COS 1 cells would be larger than the molecular weight presumable from the size of the peptide chain because of the addition of sugar chain. In consequence, as a first step, a partially purified TPO fraction was prepared in the following manner from a culture supernatant containing the human complete length TPO (derived from the expression plasmid pHTP1, F1 clone) expressed in COS 1 cells. That is, using a developing solvent A (0.1% trifluoroacetic acid (TFA))

and a developing solvent B (1-propanol containing 0.05% TFA), a 0.3 ml portion of the culture supernatant was applied to YMC-Pack PROTEIN-RP (0.46 cm in diameter and 15 cm in bed height, manufactured by YMC, catalog No. A-PRRP-33-46-25) column which has been equilibrated in advance with 25% B, 25% B was passed through the column for 5 minutes at a flow rate of 0.4 ml/min and then fractionation was effected by a linear density gradient of from 25% B to 50% B spending 50 minutes. TPO activity was found in eluates within the range of 34.5% and 43.5% as propanol concentration, and these eluates were evaporated to dryness by centrifugation evaporation to obtain a partially purified sample.

Next, TPO activity was examined by the M-07e assay system after extracting protein from a gel of SDS-PAGE electrophoresis carried out under a non-reducing condition as described in Example 1, or molecular weight was measured based on reduction-treated DPCIII markers by means of a western analysis which will be described later in Example 45. As a result, TPO activity was found within a broad apparent molecular weight range of approximately from 69,000 to 94,000, thus confirming variation in the molecular weight. Also, apparent molecular weight of TPO based on reductiontreated DPCIII markers after its N-glycosidic linkage type sugar chain digestion with N-glycanase (manufactured by Genzyme, catalog No. 1472-00) was measured to be 36,000 to 40,000 which was larger than the molecular weight of about 35,000 presumable from the size of the peptide chain, thus strongly indicating the presence of an O-glycosidic linkage type sugar chain too.

In the same manner, apparent molecular weight of the human complete length TPO (derived from the expression plasmid pHTP1, P1 clone) expressed in COS 1 cells was found to be 63,000 to 83,000. <Example 38>

### Biological characteristics of human TPO

Effects of human TPO on human and rat hematopoietic cells were examined, mainly using culture supernatant from COS 1 cells transfected with pHTF1.

In the colony assay using CD34+DR+ cell fraction prepared from human cord blood, human TPO stimulated the formation of significant number of megakaryocyte colonies. For example, 11.5 megakaryocyte colonies were formed by 6,000 CD34+DR+ cells in the presence of 10% (v/v) of culture supernatant from COS1 cells transfected with pHT1-231. To examine the effect of human TPO on human megakaryocyte progenitor cells in peripheral blood, CD34+ cells were prepared from human peripheral blood as follows. A leukocyte fraction obtained from human peripheral blood by a Ficoll-Paque density gradient was depleted of cells adherent to plastic dishes. The nonadherent cells, subsequently, were depleted of SBA (soybean agglutinin) positive cells by a panning using AIS Microselecter SBA(Asahi Medical). The resultant cells were incubated on AIS Microselecter CD 34, and the adsorbed cells, CD 34+ cells, were collected when CD3+ cells were cultured for 10 days in the presence of culture supernatant from transfected COS 1 cells in a liquid medium, selective proliferation of large size Gpllb/Illa+ cells was observed, and increase in the ploidy was found in these Gpllb/Illa+ cells. These results strongly suggested that human TPO exerts its selective action on progenitor cells of human megakaryocyte lineage and stimulates their proliferation and differentiation.

In the colony assay using GpIIb/IIIa+ cells highly enriched for CFU-MK from rat bone marrow cells and plastic nonadherent cells obtained at the preceding step of GpIIb/IIIa+ cells, human TPO induced the formation of a significant number of megakaryocyte colonies. For example, 34.5 megakaryocyte colonies were formed by 1,000 GpIIb/IIIa+

cells and 28.5 megakaryocyte colonies were formed by 20,000 plastic nonadherent cells in the presence of 20% (v/v) of culture supernatant from transfected COS 1 cells. In addition, although the plastic nonadherent cell fraction contains various hematopoietic progenitor cells other than CFU-MK, only megakaryocyte colonies were formed in the presence of human TPO, strongly suggesting that human TPO exerts its selective action on progenitor cells of the megakaryocyte lineage. When the GpIIb/IIIa+ cells obtained from rat bone marrow was cultured for 3 to 5 days in the presence of 20% (v/v) of culture supernatant from transfected COS 1 cells in a liquid culture, increase in the ploidy was observed clearly on 5 days of the culture.

#### <Example 39≥

Construction of vector for use in the expression of a fusion protein (to be referred to as "GST-TPO(1-174)" hereinafter) of glutathione-S-transferase (GST) and human TPO (amino acid residues 1 to 174) in *E. coli* 

In order to facilitate expression of human TPO in *E. coli*, an artificial gene which encodes human TPO and contains an *E. coli* preference codon was prepared. The nucleotide sequence of this DNA contains an amino-terminal methionine codon (ATG) at the -1 position for use in the translation initiation in *E. coli*.

Synthetic oligonucleotides 1 to 12:

1:5-CTAGAAAAAACCAAGGAGGTAATAAATAATGAGCCC

GGCTCCGCCAGCTTGTGACCTTCGTGTTCTGTCT-3' (SEQ ID NO:83);

2:5-CAGTTTAGACAGAACACGAAGGTCACAAGCTGGCGG

AGCCGGGCTCATTATTTATTACCTCCTTGGTTTTTT-3' (SEQ ID NO:84);

3: 5-AAACTGCTTCGCGACTCTCACGTGCTGCACTCTCG

TCTGTCCCAGTGCCCGGAAGTTCACCCGCTGCCG-3' (SEQ ID NO:85);

4: 5-CGCGCTCGCCACCGCGCTGAACTTCCGGCCACTGGG

ACAGACGAGAGTGCAGCACGTGAGAGTCGCGAAG-3 (SEQ ID NO:86);

5: 5-ACCCCGGTTCTGCTTCCGGCTGTCGACTTCTCCCT

GGGTGAATGGAAAACCCAGATGGAAGAGACCAAA-3 (SEQ ID NO87);

6: 5-CTGAGCTTTGGTCTCTTCCATCTGGGTTTTCCATT

CACCCAGGGAGAAGTCGACAGCCGGAAGCAGAAC-3' (SEQ ID NO 88);

7: 5-GCTCAGGACATCCTGGGTGCAGTAACTCTGCTTCT

GGAAGGCGTTATGGCTGCACGTGGCCAGCTTGGC-3' (SEQ ID NO:89);

8: 5-GGTCGGGCCAAGCTGGCCACGTGCAGCCATAACGC

CTTCCAGAAGCAGAGTTACTGCACCCAGGATGTC-3' (SEQ ID NO:90);

9: 5-COGACCTGCCTGTCTTCCCTGCTTGGCCAGCTGTC

TGGCCAGGTTCGTCTGCTGCTCGCGCGCTCTGCAG-3' (SEQ ID NO.91);

10:5-CAGAGACTGCAGAGCGCCGAGCAGACGAACCTGGC

CAGACAGCTGGCCAAGCAGGGAAGACAGGCA-3' (SEQ ID NO.92);

11:5-TCTCTGCTTGGCACCCAGCTGCCGCCACAGGGCCG

TACCACTGCTCACAAGGATCCGAACGCTATCTTCC-3' (SEQ ID NO:93); and 12:5-AAGACAGGAAGATAGCGTTCGGATCCTTGTGAGCA

GTGGTACGGCCCTGTGGCGGCAGCTGGGTGCCAAG-3 (SEQIDNO94)

were prepared and each of the synthetic oligonucleotides 2 to 11 was .phosphorylated with T4 kinase (manufactured by Pharmacia) in a solution composed of 1 mM ATP, 10 mM Trisacetate, 10 mM Mg-acetate and 50 mM K-acetate. In order to make these synthetic oligonucleotides into 6 double-stranded DNA fragments, these synthetic oligonucleotides were made into combinations of 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10 and 11 and 12, and each combination was subjected to annealing in a solution composed of 10 mM Tris/HCI (pH 7.5), 10 mM MgCl<sub>2</sub> and 50 mM NaCl. Next, 3 double-stranded DNA fragments of 1 and 2, 3 and 4, and 5 and 6 and the other 3 double-stranded DNA fragments of 7 and 8, 9 and 10 and 11 and 12 were respectively treated with T4 ligase (manufactured by Life Technology), and these 2 reaction solutions were again treated with T4 ligase in the same manner. The DNA fragment obtained by the ligation reaction was digested with BamHI (manufactured by Boehringer-Mannheim) and subjected to 2% agarose gel electrophoresis to recover a fragment of about 390 to 400 bp in size which was subsequently purified using Prep-A-Gene DNA Purification Kit and sub-cloned into pUC18 which has been digested in advance with Xbal and BamHI (E. coli DH5 $\alpha$  was used as the host). From the clones thus obtained, a clone having the artificial gene which encodes human TPO and contains the following E. coli preference codons was selected by nucleotide sequence analysis and named pUC18(XB)(1-123). DNA sequence of the coding chain is shown in the Sequence Listing (SEQ ID NO:9).

CTAGAAAAAA CCAAGGAGGT AATAAATAAT GAGCCCGGCT CCGCCAGCTT GTGACCTTCG TTTTTT GGTTCCTCCA TTATTTATTA CTCGGGCCGA GGCGGTCGAA CACTGGAAGC XbaI TGTTCTGTCT AAACTGCTTC GCGACTCTCA CGTGCTGCAC TCTCGTCTGT CCCAGTGCCC ACAAGACAGA TITGACGAAG CGCTGAGAGT GCACGACGTG AGAGCAGACA GGGTCACGGG 5 160 GGAAGTTCAC CCGCTGCCGA CCCCGGTTCT GCTTCCGGCT GTCGACTTCT CCCTGGGTGA CCTTCAAGTG GGCGACGGCT GGGGCCAAGA CGAAGGCCGA CAGCTGAAGA GGGACCCACT 7 230 ATGGAAAACC CAGATGGAAG AGACCAAAGC TCAGGACATC CTGGGTGCAG TAACTCTGCT TACCTTTTGG GTCTACCTTC TCTGGTTTCG AGTCCTGTAG GACCCACGTC ATTGAGACGA TCTGGAAGGC GTTATGGCTG CACGTGGCCA GCTTGGCCCG ACCTGCCTGT CTTCCCTGCT AGACCTTCCG CAATACCGAC GTGCACCGGT CGAACCGGGC TGGACGGACA GAAGGGACGA TGGCCAGCTG TCTGGCCAGG TTCGTCTGCT GCTCGGCGCT CTGCAGTCTC TGCTTGGCAC ACCGGTCGAC AGACCGGTCC AAGCAGACGA CGAGCCGCGA GACGTCAGAG ACGAACCGTG 11 370 380 390 400 410 420

CCAGCTGCCG CCACAGGGCC GTACCACTGC TCACAAGGAT CCGAACGCTA TCTTCC

GGTCGACGGC GGTGTCCCGG CATGGTGACG AGTGTTCCTA GGCTTGCGAT AGAAGGACAG AA

12 BAMHI

Single-stranded cDNA was synthesized from 1 µg of human normal liver-derived poly (A)+ RNA using oligo dT primer, and PCR was carried out using 0.1 volume of the cDNA synthesis reaction solution as a template. The PCR reaction (incubation at 96°c for 2 minutes, repetition of a total of 30 cycles of reaction, each cycle consisting of incubations at 95 c for 1 minute, at 58 c for 1 minute and at 72 c for 1 minute, and final incubation at 72°c for 7 minutes) was carried out with a volume of 100 µl using hTPO-C and hTPO-Z(EcoRI) as primers. The human TPO cDNA fragment obtained in this way was digested with BamHI and EcoRI and subjected to 2% agarose gel electrophoresis to recover a fragment of about 600 bp in size which was subsequently purified using Prep-A-Gene DNA Purification Kit and sub-cloned into pUC18 which has been digested in advance with EcoRI and BamHI (E. coli DH5a was used as the host). A clone encoding correct human TPO nucleotide sequence was selected by nucleotide sequence analysis and named pUC18(BE)(124-332). Oligonucleotide sequences of the primers used in the PCR reaction are as follows:

hTPO-C: 5'-GGA GGA GAC CAA GGC ACA GGA-3' (SEQ ID NO:95) (positions 329 to 349 of pHTF1 clone); and

hTPO-Z(*EcoRI*): 5'-CCG GAA TTC TTA CCC TTC CTG AGA CAG ATT-3' (SEQ ID NO:96) (prepared by adding an *EcoRI* recognition sequence to an antisense primer corresponding to positions 1,143 to 1,163 of pHTF1 clone).

The clone pUC18(BE)(124-332) was digested with BamHI and EcoRI and subjected to 2% agarose gel

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electrophoresis to recover a human TPO cDNA C-terminal side fragment of about 600 bp in size which was subsequently purified using Prep-A-Gene DNA Purification Kit and subcloned into pUC18(XB)(1-123) which has been digested in advance with *EcoRI* and *BamHI* (*E. coli* DH5 $\alpha$  was used as the host). A clone obtained in this manner was named pUC18(XE)(1-332).

Since the presence of human TPO activity in the human TPO peptide fragment of 1 to 163 position amino acid residues had been confirmed by an example in which various C-terminal deletion constructs of human TPO cDNA were prepared and expressed to measure *in vitro* human TPO activity, an expression vector containing this peptide fragment was constructed. In this case, expression of the DNA sequence which encodes GST-TPO(1-174) was carried out.

Since a nucleotide sequence corresponding to positions 681 to 686 (amino acid residues of positions 173 and 174) of the pHTF1 clone is recognized by a restriction enzyme Sacl, two terminal codons were introduced using 2 synthetic oligonucleotides making use of the recognition site of this That is, two synthetic oligonucleotides restriction enzyme. SSE1 and SSE2 were annealed in a solution composed of 10 mM Tris/HCI (pH 7.5), 10 mM MgCl2 and 50 mM NaCl, and, making use of DNA Ligation Kit (manufactured by Takara Shuzo), the thus obtained double-stranded DNA fragment was introduced into pUC18(XE)(1-332) from which the human TPO cDNA Cterminal fragment of about 480 bp has been removed by its digestion with Sacl and EcoRI, thereby obtaining a clone pUC18(XS)(1-174) (E. coli DH5 $\alpha$  was used as the host). Sequences of the synthetic oligonucleotides used herein are as follows:

SSE1: CTAATGAG (SEQ ID NO:97);

SSE2: AATTCTCATTAGAGCT (SEQ ID NO:98);

SACI SSE1 ECORI

5'-CTAATGAG-3' (SEQ ID NO:99)

3'-TCGAGATTACTCTTAA-5' (SEQ ID NO:100)

SSE2

The clone pUC18(XS)(1-174) obtained above was digested with Xbal and EcoRl and subjected to 2% agarose gel electrophoresis to recover a fragment of about 600 bp in size which encodes amino acid residues 1 to 174 of human TPO, and the fragment was subsequently purified using Prep-A-Gene DNA Purification Kit and sub-cloned into pBluescript IISK+ (manufactured by Stratagene) which had been digested in advance with Xbal and EcoRl (E. coli DH5a was used as the host). A clone obtained in this way was named pBL(XS)(1-174). in the same manner, the clone pBL(XS)(1-174) was digested with Xbal and HindIII to recover a fragment of about 550 bp in size which encodes amino acid residues 1 to 174 of human TPO, and the fragment was subsequently purified using Prep-A-Gene DNA Purification Kit and sub-cloned into pCFM536 (EP-A-136490) which had been digested in advance with Xbal and HindIII (E. coli DH5 $\alpha$  was used as the host). A clone obtained in this way was named pCFM536/hT(1-174).

The following experiment was carried out in order to effect expression of GST-TPO(1-174) making use of pGEX-2T (manufactured by Pharmacia) which is an expression vector of glutathione-S-transferase (GST) fusion protein. PCR reaction (incubation at 96°c for 2 minutes, repetition of a total of 22 cycles of reaction, each cycle consisting of incubations at 95°c for 1 minute, at 41°c for 1 minute and at 72°c for 1 minute, and final incubation at 72°c for 7 minutes) was carried out using pCFM536/hT(1-174) as a template and two PCR primers GEZ1 and GEX3. The human TPO encoding fragment obtained in this way was digested with Nael and

EcoRI and subjected to 2% agarose gel electrophoresis to recover a fragment of about 550 bp in size, the fragment was subsequently purified using Prep-A-Gene DNA Purification Kit and cloned into pGEX-2T which has been digested in advance with EcoRI and Smal (E. coli DH5 was used as the host) and then a clone, named pGEX-2T/hT(1-174), which encodes correct human TPO nucleotide sequence was selected by nucleotide sequence analysis to prepare a transformant for use in the expression of GST-TPO(1-174). Oligonucleotide sequences of the primers used in the PCR reaction are as follows:

GEX1: 5'-ATC GCC GGC TCC GCC AGC TTG TGA C-3' (SEQ ID NO:101) (prepared by adding a *Nael* recognition sequence to the sequence of positions 21 to 39 in SEQ ID NO:10); and

GEX3: 5'-GCC GAA TTC TCA TTA GAG CTC GTT CAG TGT-3' (SEQ ID NO:102) (an antisense primer corresponding to the sequence of positions 523 to 549 in SEQ ID NO:10).

This expression plasmid contains a DNA sequence which encodes GST protein followed by a thrombin recognition peptide and a human TPO (amino acid residues 1 to 174). The DNA sequence which encodes thrombin recognition peptide and human TPO (amino acid residues 1 to 174) are shown in the Sequence Listing (SEQ ID NO:10).

## <Example 40>

## Expression of GST-TPO(1-174) in E. coli

The transformant prepared in Example 39 was cultured overnight at 37 °c on a shaker using 60 ml of LB medium containing 50  $\mu$ g/ml of ampicillin, and 25 ml portion of the resulting culture broth was added to 1,000 ml of LB medium containing 50  $\mu$ g/ml of ampicillin and cultured at 37 °c on the shaker until OD at A<sub>600</sub> reached 0.7 to 0.8. Thereafter, IPTG was added to the culture broth to a final concentration of 0.1 mM and the shaking culture was continued

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for additional 3 hours in order to induce expression of GST-TPO(1-174).

# <Example 41> Purification of GST-TPO(1-174) expressed in E. coli. and confirmation of its biological activity

A 5.9 g portion of frozen cells of the recombinant strain which produces GST-TPO(1-174) derived from human TPO nucleotide sequence-encoding clone pGEX-2T/hT(1-174) were suspended in 10 ml of water and disruptured using a high By subjecting the suspension to pressure disintegrator. centrifugation, GST-TPO(1-174) was recovered in the precipitated fraction and most parts of contaminated proteins, cell components and the like were removed. Next, the thus recovered precipitated fraction containing GST-TPO(1-174) was suspended in 5 ml of water to which were subsequently added, with stirring, 6 ml of 1 M Tris buffer (pH 8.5), 120 ml of 10 M urea and 16 ml of water. After 5 minutes of stirring to solubilize the contents, the resulting solution was evenly divided into 4 portions to carry out the following steps (1) to (4).

(1) One of the divided samples was diluted by a factor of 10 with 20 mM Tris buffer having a pH value of 8.5. To this were added reduced type glutathione and oxidized type glutathione to respective final concentrations of 5 mM and 0.5 mM, followed by overnight incubation at 4°c. The thus prepared mixture was subjected to centrifugation to recover GST-TPO(1-174) as the supernatant fluid which was subsequently diluted by a factor of 2 with 20 mM sodium citrate buffer having a pH value of 5.5 and then adjusted to pH 5.5 with acetic acid. GST-TPO(1-174) in the solution was applied to SP Sepharose Fast Flow (manufactured by Pharmacia Biotech, catalog No. 17-0729-01) cation exchange column which has been equilibrated in advance with 20 mM sodium

citrate buffer pH 5.5. After washing the column with 20 mM sodium citrate buffer pH 5.5, elution of GST-TPO(1-174) was effected using 20 mM sodium citrate buffer pH 5.5 containing 500 mM sodium chloride. A 129 ml portion of the eluate was mixed with 2.6 ml of 1 M Tris buffer pH 8.5, and the resulting mixture having a pH value of 8.1 was applied to Glutathione Sepharose 4B (manufactured by Pharmacia Biotech, catalog No. 17-0756-01) column to adsorb GST-TPO(1-174). washing the column with PBS, elution of GST-TPO(1-174) was effected using 20 mM Tris buffer pH 8.5 containing 10 mM of reduced type glutathione. The resulting eluate was mixed with 37 NIH units of thrombin, allowed to stand for 4 hours at room temperature, diluted with PBS by a factor of 10 and then applied to Glutathione Sepharose 4B column to adsorb digested GST and to recover TPO(1-174) in the non-adsorbed fraction. The thus recovered non-adsorbed fraction was diluted by a factor of 3 with 20 mM sodium citrate buffer pH 5.5 and applied to SP Sepharose Fast Flow cation exchange column which has been equilibrated in advance with the same buffer, and the compound of interest was eluted with a linear density gradient of 0 to 500 mM sodium chloride dissolved in the same buffer.

- (2) All operations of the step (1) were carried out in the presence of 0.1% polysorbate 80.
- (3) One of the divided samples was diluted by a factor of 10 with 20 mM Tris buffer having a pH value of 8.5. To this were added reduced type glutathione and oxidized type glutathione to respective final concentrations of 5 mM and 0.5 mM, followed by overnight standing at 4 °C. The thus prepared mixture was subjected to centrifugation to recover GST-TPO(1-174) as the supernatant fluid which was subsequently diluted by a factor of 2 with 20 mM sodium citrate buffer having a pH value of 5.5 and then adjusted to pH 5.5 with acetic acid. GST-TPO(1-174) in the solution was applied to SP

Sepharose Fast Flow cation exchange resin which has been equilibrated in advance with 20 mM sodium citrate buffer pH 5.5. After washing the resin with 20 mM sodium citrate buffer pH 5.5, elution of GST-TPO(1-174) was effected using 20 mM sodium citrate buffer pH 5.5 containing 500 mM sodium chloride. The eluate was adjusted to pH 8 with 1 M Tris buffer pH 8.5, mixed with 320 NIH units of thrombin to remove GST polypeptide sequences via cleavage at a thrombin recognition peptide linker, allowed to stand for 4 hours at room temperature, diluted by a factor of 5 with 20 mM sodium citrate buffer pH 5.5 and then applied to SP Sepharose Fast Flow cation exchange column which has been equilibrated in advance with the same buffer, and the compound of interest was subsequently eluted with a linear density gradient of 0 to 500 mM sodium chloride sissolved in the same buffer.

(4) All operations of the step (3) were carried out in the presence of 0.1% polysorbate 80.

Each of the fractions eluted at sodium chloride densities of about 200 to 400 mM by the SP Sepharose Fast Flow cation exchange column chromatography was thoroughly dialyzed against IMDM culture medium and then evaluated by the rat CFU-MK assay system. When the fraction was analyzed by SDS-PAGE in the presence of a reducing agent, a band corresponding to a molecular weight of 19 kd was detected (purity, 1 to 20%) as one of the main protein bands of the fraction. When N-terminal sequence analysis of this band was carried out by subjecting it to SDS-PAGE and PVDF membrane transfer in accordance with the procedure described in Example 1, it was confirmed that this band contains a TPO sequence to be expressed as the pGEX-2T/hT(1-174)-derived fusion protein: The deduced amino acid sequence of the resulting TPO was [Gly-1]TPO given the sequence of the thrombin recognition peptide linker and the known activity of thrombin on the linker.

<Example> 42
Construction of vector for use in E. coli expression of human TPO (amino acid residues 1 to 163) having substitutions in the position 1 (Ser -> Ala) and position 3 (Ala -> Val)

The clone pUC18(XE)(1-332) prepared in Example 39 was digested with Xbal and EcoRl and subjected to 2% agarose gel electrophoresis to recover a fragment of about 1,000 bp in size which encodes amino acid residues 1 to 332 of human TPO, and the fragment was subsequently purified using Prep-A-Gene DNA Purification Kit and sub-cloned into pBluescript II SK+ (manufactured by Stratagene) which has been digested in advance with Xbal and EcoRl (E. coli DH5a was used as the host). A clone obtained in this way was named pBL(XE)(1-332). Next, a region of the clone pBL(XE)(1-332) from its BamHI recognition sequence to position 163 amino acid (positions 366 to 489) was changed to E. coli dominant codons. Synthetic oligonucleotides 13 to 20 shown below were prepared, and each pair of synthetic oligonucleotides 13 and 14, 15 and 16 and 17 and 18 were phosphorylated in the same tube containing a solution composed of 0.1 mM ATP, 10 mM Tris-acetate, 10 mM Mg-acetate and 50 mM K-acetate. After further addition of 1/10 volume of a solution composed of 100 mM Tris/HCI (pH 7.5), 100 mM MgCl<sub>2</sub> and 500 mM NaCl, the resulting solution was heated in a boiling water bath for 3 minutes and then allowed to cool to room temperature to obtain a doublestranded DNA fragment. Next, the thus obtained three pairs of double-stranded DNA fragments consisting of 13 and 14, 15 and 16 and 17 and 18 were ligated using DNA Ligation Kit (manufactured by Takara Shuzo), and PCR was carried out using the thus ligated product as a template and the synthetic oligonucleotides 19 and 20 as primers. The thus obtained PCR product was digested with BamHI and HindIII and subjected to

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2% agarose gel electrophoresis to recover a fragment of about 130 bp in size using Prep-A-Gene DNA Purification Kit. This was sub-cloned into pBL(XE)(1-332) which has been digested in advance with *Bam*HI and *HindIII* (*E. coli* DH5 was used as the host). A clone having the following nucleotide sequences was selected from the thus obtained clones by sequence analysis and named pBL(XH)(1-163):

13: 5-GATCCGAACGCTATCTTCCTGTCTTTCCAGCACCTGCTGCGT-3'

(SEQ ID NO:103);

14: 5-TTTGCCACGCAGCAGGTGCTGGAAAGACAGGAAGATAGOGTTCG-3

(SEQ ID NO:104);

15: 5-GGCAAAGTTCGTTTCCTGATGCTGGTTGGCGGTTCTACCCTG-3'

(SEQ ID NO:105);

16: 5-ACGCACAGGGTAGAACCGCCAACCAGCATCAGGAAACGAAC-3'

(SEQ ID NO:106);

17: 5-TGCGTTCGTCGGGCGCCGCCAACCACTGCTGTTCCGTCTTAATGAA-3"

(SEQ ID NO:107);

18: 5-AGCTTTCATTAAGACGGAACAGCAGTGGTTGGCGGCGCCCGACGA-3"

(SEQ ID NO:108);

19: 5'-AAGGATCCGAACGCTATCTTCCTG-3' (SEQ ID NO:109); and

20: 5'-AGAAGCTTTCATTAAGACGGAACA-3' (SEQ ID NO:110).

For the purpose of increasing expression quantity of human TPO (positions 1 to 163) and preventing hydrolysis of N-terminal by protease, an expression vector was constructed for use in the expression of a mutation type human TPO (positions 1 to 163) (to be referred to as "h6T(1-163)" hereinafter) having substitutions at the position 1 (Ser to Ala) and position 3 (Ala to Val) of human TPO (positions 1 to 163) ([Ala<sup>1</sup>, Val<sup>3</sup>]TPO(1-163)) and, at the same time, encoding Lys at the -1 position and Met at the -2 position ([Met-<sup>2</sup>, Lys-<sup>1</sup>, Ala<sup>1</sup>, Val<sup>3</sup>]TPO(1-163)). Four synthetic oligonucleotides shown below were prepared, and synthetic oligonucleotides 2-9 and 3-3 were phosphorylated with T4 kinase (manufactured)

by Pharmacia) in a solution composed of 1 mM ATP, 10 mM Tris-acetate, 10 mM Mg-acetate and 50 mM K-acetate. In order to make these synthetic oligonucleotides into double-stranded DNA fragments, each pair of the single-stranded DNA fragments 1-9 and 2-9 and 3-3 and 4-3 were annealed in a solution composed of 10 mM Tris/HCl (pH 7.5), 10 mM MgCl<sub>2</sub> and 50 mM NaCl. Next, the thus obtained two pairs of double-stranded DNA fragments consisting of 1-9 and 2-9 and 3-3 and 4-3 were treated with DNA Ligation Kit (manufactured by Takara Shuzo). The DNA fragment thus obtained by the ligation reaction was sub-cloned into pBL(XH)(1-163) which has been digested in advance with Xbal and Nrul, and a clone correctly substituted by the following synthetic oligonucleotide was selected by sequence analysis (E. coli DH5α was used as the host) and named pBL(XH)h6T(1-163):

```
1-9: 5'-CTAGAAAAAACCAAGGAGGTAATAAATAATGAAAGCACCTGTACCA-3'
                                                     (SEQ ID NO:111);
2-9: 5'-CAGGTGGTACAGGTGCTTTCATTATTATTACCTCCTTGGTTTTTT-3'
                                                     (SEQ ID NO:112);
3-3: 5'-CCTGCATGTGATTTACGGGTCCTGTCTAAACTGCTGCG-3'
                                                     (SEQ ID NO:113);
4-3: 5'-CGCAGCAGTTTAGACAGGACCCGTAAATCACATG-3'
                                                     (SEQ ID NO:114);
                                            40
                                30
         10
              1-9
                     20
 CTAGAAAAA CCAAGGAGGT AATAAATAAT GAAAGCACCT
     TTTTTT GGTTCCTCCA TTATTTATTA CTTTCGTGGA
              2-9
<u>XbaI</u>
                                                              80
                           50
                                       60 <u>3-3</u>
```

GTACCACCTG CATGTGATTT ACGGGTCCTG TCTAAACTGC TGCG CATGGTGGAC GTACACTAAA TGCCCAGGAC AGATTTGACG ACGC 4-3

(SEQ ID NO:115)

The clone pBL(XH)(1-163) was digested with Xbal and HindIII to recover a fragment of about 500 bp in size which encodes amino acid residues 1 to 163 of the mutation type human TPO, and the fragment was subsequently purified using Prep-A-Gene DNA Purification Kit and cloned into pCFM536 (EP-A-136490) which has been digested in advance with Xbal and HindIII (E. coli JM109 which has been transformed in advance with pMW1 (ATCC No. 39933) was used as the host). A clone obtained in this way was named pCFM536/h6T(1-163), and an E. coli strain having this expression vector was used as a transformant for use in the expression of the mutation type human TPO, namely h6T(1-163). This expression plasmid contains the DNA sequence shown in the Sequence Listing (SEQ ID NO:11).

### <Example 43>

#### Expression of h6T(1-163) in E. coli

Expression of the expression plasmid pCFM536 is controlled by  $\lambda PL$  promoter which by itself is under control of cl857 repressor gene. The transformant obtained in <Example 42> was cultured overnight at 30°c on a shaker using 60 ml of LB medium containing 50  $\mu g/ml$  of ampicillin and 12.5  $\mu g/ml$  of tetracycline, and 25  $\mu l$  portion of the resulting culture broth was added to 1,000 ml of LB medium containing 50  $\mu g/ml$  of ampicillin and cultured at 37°c on the shaker until OD at  $A_{600}$  reached 1.0 to 1.2. Thereafter, about 330 ml of LB medium heated to 65°c was added to the culture broth to adjust the final medium temperature to 42°c, and the shaking culture was continued for 3 hours at 42°c to induce expression of h6T(1-163).

<Example 44>
Purification of h6T(1-163) expressed in

## E. coli. and confirmation of its biological activity

A 3.6 g portion of frozen cells of the h6T(1-163) producing recombinant strain were suspended in 10 ml of water and disruptured using a high pressure disintegrator. By subjecting the suspension to centrifugation, the precipitated fraction was recovered and most parts of contaminated proteins, cell components and the like were removed. The thus recovered precipitated fraction containing h6T(1-163) was suspended in 7 ml of water, and 3 ml of 1 M Tris buffer (pH 8.5) was added to the suspension with stirring, followed by further addition of urea (final concentration, 8 M), guanidine hydrochloride (final concentration, 6 M) and sodium N-lauroyl sarcosinate (final concentration, 2%). After 5 to 20 minutes of stirring at room temperature to solubilize the contents, the resulting solution was diluted 10 times with 20 mM Tris buffer having a pH value of 8.5 and subjected to protein refolding by overnight oncubation at 4°c. In this case, glutathione and copper sulfate were used as additives in addition to air oxidation. By centrifugation, h6T(1-163) was recovered in the supernatant fluid. When each of the recovered fractions was analyzed by SDS-PAGE in the presence of a reducing agent, a band corresponding to a molecular weight of 18 kd was detected (purity, 30 to 40%) as the main protein band of each fraction. When N-terminal sequence analysis of this band was carried out by subjecting it to SDS-PAGE and PVDF membrane transfer according to the procedure described in Example 1, it was confirmed that this band contains a TPO sequence to be expressed as the pCMF536/h6T(1-163)-derived mutation type TPO. When each fraction was thoroughly dialyzed against IMDM culture medium and evaluated by the rat CFU-MK assay system, TPO activity was found in all fractions in a dose-dependent fashion.

## Preparation of anti-TPO peptide antibody and detection of TPO by SDS-PAGE and western analysis

Success in preparing anti-TPO peptide antibody will render possible detection of TPO protein by immunological means. In consequence, three regions which appear to be relatively useful as antigens (see below) were selected from the confirmed TPO amino acid sequences, a quadruple-stranded multiple antigen peptide (MAP) type peptide was synthesized in accordance with the procedure of Tam (*Proc. Natl. Acad. Sci. USA*, 85, 5409 - 5413, 1988) using each of the selected regions, and then each of 2 rabbits was immunized 8 times with 100 µg of each of the thus synthesized peptides to obtain respective antiserum samples.

Amino acid sequences contained in the synthesized peptide antigens

(a) Rat TPO(9-28) (Peptide RT1 region)
PRLLNKLLRDSYLLHRRLSQ (SEQ ID NO:116)

(R at Position 24 is originally S in the amino acid residues determined from the gene.)

- (b) Rat TPO(46-66) (Peptide RT2 region)
  FSLGEWKTQTEQSKAQDILGA (SEQ ID NO:117)
- (c) Rat TPO(163-180) (Peptide RT4 region) SRTSQLLTLNKFPNRLLD (SEQ ID NO:118)

(LLD at Positions 178-180 is originally TSG in the amino acid residues determined from the gene.)

Next, of these antiserum samples, anti-RT1 peptide and anti-RT2 peptide were firstly subjected to a protein A (PROSEP-A; manufactured by Bioprocessing Ltd., catalog No. 8427) column chromatography to prepare IgG fractions (2,344 mg and 1,920 mg, respectively), and 54 mg and 32 mg portions of the respective fractions were biotinylated by their coupling with active type biotin (NHS-LC-Biotin II; manufactured by

PIERCE, catalog No. 21336). A recombinant TPO-containing sample was subjected to SDS-PAGE and then to electroblotting on PVDF or nitrocellulose membrane in the same manner as described in Example 1, and western analysis was carried out in the usual way using these biotinylated antibodies as first antibodies.

That is, the membrane after blotting was washed with a solution composed of 20 mM Tris-HCl and 0.5 M NaCl (pH 7.5) (TBS) for 5 minutes and twice with 0.1% Tween 20containing TBS (TTBS) for 5 minutes each and then treated with a blocking agent (BlockAce; manufactured by Dainippon Pharmaceutical, catalog No. uk-B25) for 60 minutes. Next, the membrane was treated for 60 minutes with TTBS solution containing 10 µg/ml of biotinylated anti-TPO peptide antibody. 0.05% BSA and 10% BlockAce and then washed twice with TTBS for 5 minutes each. Thereafter, the membrane was treated for 30 minutes with a solution prepared by diluting alkaline phosphatase-labeled avidin (manufactured by Leinco Technologies, catalog No. A108) by a factor of 5,000 with TTBS solution containing 10% BlockAce and washed twice with TTBS for 5 minutes each and with TBS for 5 minutes, and then color development was effected by the use of an alkaline phosphatase substrate (manufactured by Bio-Rad, catalog No. 170-6432). The western analysis described above was carried out at room temperature.

As the results, it was able to recognize not only various recombinant rat TPO proteins expressed in COS 1 cells but also various recombinant human TPO proteins expressed in COS 1 cells and *E. coli* cells (illustratively, plasmid pHTP1- or plasmid pHTF1-derived human TPO expressed in COS 1 cells and N-glycosidic linkage-cleaved product thereof, GST-TPO(1-174) expressed in *E. coli* and thrombin-digested product thereof and h6T(1-163) which is a mutation type TPO expressed in *E. coli*, which have been described in Examples).

In addition, the results also have rendered possible analysis of these various types of recombinant human TPO.

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In addition to the above, possibility for the preparation of anti-human TPO peptide antibodies was confirmed. Antibodies obtained by these techniques can be applied not only to western analysis but also to purification of TPO by antibody columns and every usually used antibody-aided immunological means.

Six regions in the human TPO amino acid sequence shown in SEQ ID NO:7, which were expected to have relatively suitable antigenicity, were selected (see Table 4). Tetrameric Multiple Antigen Peptide (MAP) type peptides, which correspond to the regions, respectively, were synthesized. Each peptide was immunized to two rabbits 8 times in an amount of 100 mg/time.

Separately from this, a monomeric peptide in which a cysteine residue had been bonded to the C-terminus of each peptide region shown in Table 4 was synthesized too. This peptide was then used as a test antigen to determine an antibody titer using enzyme immunoassay. As a result, the increase of the antibody titer was confirmed for the above prepared sera, so these sera were employed as antisera.

The antibodies were prepared by immunizing each antigenic peptide to two rabbits to produce antisera directed against the peptide. The resultant two anti-HT1 peptide antibodies are distinguishably referred to as anti-HT1-1 peptide antibody and anti-HT1-2 peptide antibody depending on the rabbit individuals.

An example of the purification of the anti-HT1-1 peptide antibodies will be illustrated below.

First, 30 mg of a monomeric peptide of HT1 to which has been bonded a cystein residue was coupled to 12 ml of Sulfo-Link coupling gel (Pierce, Catalogue No. 44895). Briefly, a peptide solution containing the antigen was coupled

for 15 minutes to the gel equilibrated with a coupling buffer (50 mM Tris, 5 mM EDTA-Na pH 8.5) of 6 volumes relative to the gel volume. Then, after incubation for 30 minutes, the gel was washed with the coupling buffer of 3 volumes relative to the gel volume. The coupling buffer containing 0.05 M L-cystein-HCl was added to the gel in an amount of 1 ml/ml gel to block unreacted radicals over 15 minutes. After incubation for 30 minutes, the gel was washed with the coupling buffer of 8 volumes relative to the gel volume. All the coupling reactions were carried out at room temperature. Thus the peptide was covalently bonded to the gel with a coupling efficiency of 28.3%, and then an antigen column with 0.8 mg of the peptide/ml gel was prepared.

76.7 ml (protein content 3620 mg) of the antiserum containing the anti-HT1-1 peptide antibody which had been bled from one of the rabbits with a total amount of 78.4 ml, was applied to the antigen column pre-equilibrated with 50 mM phosphate buffer (pH 8) containing 150 mM NaCl and 0.05% sodium azid, and then washed with the same buffer to afford 105.9 ml of a flow-through fraction (protein content 3680 mg). Then the adsorbed fraction was eluted with 0.1 M citrate buffer (pH 3.0) and immediately neutralized with 21.1 ml of 0.1 M carbonate buffer (pH 9.9), followed by concentration by means of ultrafiltration (using Amicon YM 30 membran) to give the purified anti-HT1-1 peptide antibody in a solution of 50 mM phosphate buffer (pH 8) containing 150 mM NaCl and 0.05% NaN<sub>3</sub>.

Similarly, the following antibodies were prepared: anti-HT1-2 peptide antibody (60.0 mg); anti-HT2-1 peptide antibody (18.8 mg); anti-HT2-2 peptide antibody (8.2 mg); and the like. Also, anti-HT3 to -HT6 peptide antibodies could be prepared in the similar manner.

Three mg of affinity-purified anti-HT1-1 peptide antibody, anti-HT1-2 peptide antibody, anti-HT2-1 peptide

antibody or anti-HT2-2 peptide antibody was biotinylated by coupling to an activated biotin (Pierce, NHS-LC-Biotin II, Catalogue No. 21336).

All the above purified antibodies were found to recognize and detect human TPO by Western blotting (on nitrocellulose filter or PVDF) following SDS-PAGE of a recombinant human TPO standard partially purified from the culture supernatant of CHO cells wherein a gene encoding the amino acid sequence shown in Table 4 had been introduced and expressed.

Table 4
Human TPO amino acid sequences included in the tetrameric MAP type synthetic peptide antigen

Human TPO (amino acid numbers 8-28) peptide HT1 region: DLRVLSKLLRDSHVLHSRLSQ (SEQ ID NO:119)

Human TPO (amino acid numbers 47-62) peptide HT2 region: SLGEWKTQMEETKAQD (SEQ ID NO:120)

Human TPO (amino acid numbers 108-126) peptide HT3 region: LGTQLPPQGRTTAHKDPNA (SEQ ID NO:121)

Human TPO (amino acid numbers 172-190) peptide HT4 region: NELPNRTSGLLETNFTASA (SEQ ID NO:122)

Human TPO (amino acid numbers 262-284) peptide HT5 region: SLPPNLQPGYSPSPTHPPTGQYT (SEQ ID NO:123)

Human TPO (amino acid numbers 306-332) peptide HT6 region: PSAPTPTPTSPLLNTSYTHSQNLSQEG (SEQ ID NO:124)

<Example 46>

## Preparation of anti-TPO peptide antibody column

Since the anti-rat TPO peptide antibody obtained in Example 45 was able to recognize rat and human TPO molecules, immunoglobulin (IgG) fractions of the anti-RT1 peptide antibody and anti-RT2 peptide antibody were linked to a chemically activated gel support in the following manner to prepare anti-TPO peptide antibody columns. Each of the two antibodies used as the materials was prepared from sera of two rabbits separately. That is, the anti-RT1 peptide antibody prepared from two rabbits was respectively named anti-RT1-1 peptide antibody and anti-RT1-2 peptide antibody, and the anti-RT2 peptide antibody prepared from other two rabbits was respectively named anti-RT2-1 peptide antibody and anti-Since these antibodies were RT2-2 peptide antibody. separately used for the antibody column preparation, a total of 5 columns were obtained, namely two anti-RT1 peptide antibody columns (to be referred to as "anti-RT1-1 antibody column" and "anti-RT1-2 antibody column" hereinafter), two anti-RT2 peptide antibody columns (to be referred to as "anti-RT2-1 antibody column" and "anti-RT2-2 antibody column" hereinafter) and one antibody column (anti-RT1-2 + 2-1 mix antibody column) which was prepared by mixing anti-RT1-2 peptide antibody with anti-RT2-1 peptide antibody and coupling the mixture with a gel.

Each antibody was dissolved in a solution of 50 mM Na phosphate and 0.15 M NaCl (pH 8.0) to a final concentration of 5 mg/ml (or 2.5 mg/ml each of evenly mixed anti-RT1-2 peptide antibody and anti-RT2-1 peptide antibody in the case of the anti-RT1 + 2 mix antibody column), and a 2.31 ml portion of the resulting solution was mixed with 1.54 ml in volume of a swelled formyl-activated gel (Formyl-Cellulofine, manufactured by Chisso) and subjected to 2 hours of coupling reaction at 4 °c. To this was added 1.1 ml of a 10 mg/ml solution of a reducing agent (trimethylamine borane (TMAB);

manufactured by Seikagaku Kogyo, catalog No. 680246), followed by 6 hours of additional coupling reaction and subsequent centrifugation to recover the gel portion. A 10 ml portion of purified water was added to the gel portion which was recovered again by centrifugation, and unreacted antibody molecules were removed by repeating this step 4 times. Next, the resulting gel was mixed with 4.6 ml of a blocking solution (0.2 M Na phosphate and 1 M ethanol amine, pH 7.0) and 1.1 ml of the reducing agent solution and treated at 4 c for at least 2 hours to perform blocking of active groups of unreacted gel. Thereafter, the gel was washed with purified water and DPBS using a centrifuge, packed in a small column tube, washed with a 3 M potassium thiocyanate solution and a 0.1 M glycine-HCl (pH 2.5) solution, equilibrated with DPBS and then stored.

In the 5 anti-TPO peptide antibody gels of the anti-RT1-1 antibody column, anti-RT1-2 antibody column, anti-RT2-1 antibody column, anti-RT2-2 antibody column and anti-RT1-2 + 2-1 mix antibody column, the coupling efficiency of respective IgG fractions reached 97.4%, 95.4%, 98.4%, 98.3% and 99.4%. In addition, amounts of IgG fractions coupled per gel volume were 5.6 mg/ml gel, 5.8 mg/ml gel, 5.7 mg/ml gel, 5.7 mg/ml gel and 2.9 mg/ml gel, respectively. In consequence, since it was confirmed that the coupling efficiency and coupling quantity do not significantly vary depending on the antibody origins and difference in the antigens, the experiments shown in the following Example 47 were carried out making use of these antibody columns.

Example 47>
Purification of TPO derived from culture supernatant obtained by transfecting COS 1 cells with expression vector pHTP1, using anti-TPO antibody column and then by reverse phase column chromatography, and confirmation of its biological activity

The M-07e assay system was used as the in vitro assay for the evaluation of the following purification samples. Partially purified samples of TPO derived from the culture supernatant obtained in Example 35 by transfected COS 1 cells with the expression vector pHTP1 (fractions other than the main TPO fraction, namely a passed-through fraction F1 and a fraction F3 eluted after F2 by washing with 20 mM Na citrate, pH 5.8, of Macro-Prep Methyl HIC column and fractions F1 and F3 of SP Sepharose Fast Flow column) were combined to prepare a TPO subpool fraction (5,463.79 ml; protein concentration, 0.490 mg/ml; total protein, 2,676 mg; relative activity, 12,100; and total activity, 32,380,000) concentrated using an ultrafiltration unit (Omega Ultraset, nominal molecular weight cutoff of 8,000, manufactured by Filtron), solvent of the concentrated fraction was exchanged with DPBS and then the small-volumed sample was treated with an YM-10 membrane-equipped ultrafiltration unit (manufactured by Amicon) to make it finally into 120.2 ml of a DPBS solution containing 0.05% sodium azide. Next, all of the 5 anti-TPO peptide antibody gels prepared in Example 46 were mixed and made into a single antibody column (1.6 cm in diameter and 4.8 cm in bed height, to be referred to as anti-RT1 + 2 mix antibody column), and the TPO subpool fraction was applied to the column at a flow rate of 0.033 ml/min. After completion of the sample loading, elution was carried out with DPBS to collect eluates until UV absorption became sufficiently small, and the thus collected eluates were YM-10 membrane-equipped concentrated using the ultrafiltration unit (manufactured by Amicon) to obtain a passed-through fraction F1 (82.62 ml; protein concentration, 14.3 mg/ml; total protein, 1,184 mg; relative activity, 67,500; and total activity, 79,900,000). Next, a column-adsorbed fraction F2 (92.97 ml; protein concentration, 0.12 mg/ml; total protein, 11.1 mg; relative activity, 257,100; and total activity,

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2,860,000) was eluted with an acidic eluting solution (0.1 M glycine-HCl, pH 2.5). Though the passed-through fraction F1 still contained a TPO activity, the antibody column-adsorbed TPO was further purified because it showed about 20 times increase in the relative activity. That is, using 0.1% TFA as a developing solvent A and 1-propanol containing 0.05% TFA as a developing solvent B, Capcell Pak Cl 300A (manufactured by Shiseido, catalog No. CI TYPE:SG300A; 4.6 mm in diameter and 150 mm in bed height, connected with a precolumn of 4.6 mm in diameter and 35 mm in bed height) column chromatography was carried out, by mixing the fraction F2 obtained from the antibody column with 1/10 volume of the developing solvent B and applying the mixture at a flow rate of 0.4 ml/min to the Capcell Pak Cl 300A column which has been equilibrated in advance with 20% B. After completion of the sample loading, elution was carried out for 5 minutes with 20% B and then for 50 minutes with a linear density gradient of from 20% B to 40% B, and the eluates were collected in polypropylene tubes in 1 ml (2.5 min) portions.

A 2 μl portion (1/500 fraction) of the eluate in each tube was mixed with HSA and concentrated by ultrafiltration to obtain 0.25 ml of IMDM assay culture solution containing 0.02% HSA for use in the identification of TPO active fractions by the assay. As the result, strong TPO activity was found in samples of tube Nos. 20 to 23 (within the range of 28.5 and 32.5% as propanol concentration). In addition, when these samples were subjected to western analysis in accordance with the procedure described in <Example 45>, the presence of TPO was confirmed which had an apparent molecular weight of 60,000 to 70,000 when measured under a reduced condition based on DPC III molecular weight markers, as well as the presence of other molecules having respective molecular weights of 32,000 to 43,000 and 20,000 to 30,000.

<Example 48>

Confirmation of biological activity of TPO derived from culture supernatant obtained by transfecting COS 1 cells with expression vector pHTP1 and purified up to the step of Capcell Pak CI 300A column

The TPO active fractions purified in Example 36. namely tube Nos. 20 to 23 (within the range of 28.5 and 32.5% as propanol concentration) of Capcell Pak Cl 300A column, were pooled, mixed with 0.21 ml of glycerol and then concentrated by centrifugation evaporation. This was mixed with 0.21 ml of 6 M guanidine hydrochloride solution, diluted to a volume of 1 ml with DPBS, buffer-exchanged with DPBS solution containing 0.01% HSA by Sephadex G25 column (NAP-10, manufactured by Pharmacia Biotech, catalog No. 17-0854-01) and then mixed with 1.1 ml of DPBS solution containing 0.01% HSA, thereby finally obtaining a TPO active fraction FA (2.6 ml). In order to examine biological TPO activity of this sample, in vivo assay was carried out. That is, the active fraction was subcutaneously administered to ICR male mice (8 weeks of age), each group including 4 animals, daily for 5 days at a dose of 100  $\mu$ l (having a total activity of 110,000 measured by M-07e assay system) per animal. As a control, 100 µl of DPBS containing 0.01% of HSA was subcutaneously Just before the administered in the same manner. administration and on the next day of the final administration, blood was collected from the eyeground to measure platelet counts using a microcell counter (F800, manufactured by Toa Ivo Denshi). In the TPO-treated group, platelet counts increased by a factor of about 1.42 in average after completion of the administration in comparison with the value prior to the administration and was 1.23 times higher than the case of the control group with a significant difference (p <0.05, Student ttest) between them. On the basis of these results, it was

confirmed that the aforementioned human TPO produced by mammalian cells has the ability to increase platelet counts in vivo.

<Example 49>

Confirmation of biological activity of crude TPO fraction derived from 33 liters of culture supernatant obtained by transfecting COS 1 cells with expression vector pHTP1 and partially purified by cation exchange column

(1) The M-07e assay system was used as the in vitro assay for the evaluation of the following purification samples. In the same manner as described in Example 36, a total of 33 liters of serum-free culture supernatant was obtained by transfecting COS 1 cells with the expression vector pHTP1 and filtered through a 0.22 μm filter to recover the supernatant. This was concentrated by a factor of about 10 with an ultrafiltration unit (PLGC pellicon Cassette, nominal molecular weight cutoff of 10,000, manufactured by Millipore) and mixed with about 1 mM in final concentration of a protease inhibitor p-APMSF to obtain a sample of 2,018 ml in volume (protein concentration, 3.22 mg/ml; total protein, relative activity, 66,000; total activity, 429,100,000). Next, this was concentrated repeatedly using an ultrafiltration unit (Omega Ultraset, nominal molecular weight cutoff of 30,000; manufactured by Filtron), thereby obtaining a fraction of 30,000 or more in molecular weight (volume, 1,190 ml; protein concentration, 2.54 mg/ml; total protein, 3,020 mg; relative activity, 82,500; total activity, 249,000,000) and a fraction of 30,000 or less in molecular weight (volume, 2,947 ml; protein concentration, 0.471 mg/ml; total protein, 1.402 mg; relative activity, 4,500; total activity, 6,310,000). The presence of TPO activity in the fraction of 30,000 or less in molecular weight indicates a possibility that the culture supernatant contains TPO whose molecular weight has been reduced during its expression in or secretion from the animal cells. On the other hand, the fraction of 30,000 or more in molecular weight was buffer-exchanged with 20 mM sodium citrate buffer (pH 6.1) and applied at a flow rate of 5 ml/min to SP Sepharose Fast Flow (2.6 cm in diameter and 29 cm in bed height, manufactured by Pharmacia Biotech, catalog No. 17-0729-01) column which has been equilibrated in advance with 20 mM sodium citrate buffer (pH 6.1). After completion of the sample loading, the column was washed and eluted with 20 mM sodium citrate buffer (pH 6.1). Next, using 20 mM sodium citrate buffer (pH 6.1) as a developing solvent A and a solution composed of the developing solvent A and 1 M NaCl as a developing solvent B, elution was carried out at a flow rate of 3 ml/min with a linear gradient of from 0% B to 50% B for 215 minutes and then from 50% B to 100% B for 20 minutes, and the eluates were collected in polypropylene tubes in 30 ml (10 min) portions. When a portion of each of these eluates was checked by the M-07e assay system to examine distribution of the activity, elution of TPO activity was found within a broad In consequence, fractions eluted with a NaCl concentration of 50 mM or lower, including the passed-through fraction, were pooled as a fraction F1, and the main TPO fractions eluted with 50 to 1,000 mM NaCl as a fraction F2. Volume of the fraction F1 was 1,951 ml (protein concentration, 2.05 mg/ml; total protein, 3,994 mg; relative activity, 13,500; total activity, 53,900,000) and that of F2 was 649.8 ml (protein concentration, 1.11 mg/ml; total protein, 721 mg; relative activity, 268,000; total activity, 193,000,000).

(2) Confirmation of biological activity of SP Sepharose Fast Flow fraction F2

A 2.5 ml portion of the SP Sepharose Fast Flow fraction F2 separated in the above step (1) was buffer-exchanged with 3.5 ml of DPBS solution containing 0.01% HSA

by Sephadex G25 column (NAP-25, manufactured by Pharmacia Biotech, catalog No. 17-0852-01), and biological TPO activity of this sample (protein concentration, 1.46 mg/ml) was examined in an in vivo assay. That is, the active fraction was subcutaneously administered to ICR male mice (8-weeks age), each group including 4 animals, daily for 5 consecutive days at a dose of 100 µl (having a total activity of 123,000 measured by the M-07e assay system) per animal. control, 100  $\mu$ l of DPBS containing 0.01% of HSA was subcutaneously administered in the same manner. Just before the administration and on the next day of final administration, blood was collected from the eyeground to measure platelet counts using a microcell counter (F800, manufactured by Toa lyo Denshi). In the TPO-administered group, platelet counts increased by a factor of about 1.29 in average after completion of the administration in comparison with the value prior to the administration and were 1.12 times higher than in the control group with a significant difference (p <0.05, Student t-test) These results demonstrated that the between . them. aforementioned human TPO produced by mammalian cells has . the ability to increase platelet counts.

<Example 50>
Construction of recombinant vector, pDEF202-ghTPO, for use in the expression of human TPO chromosomal DNA in CHO cells

A vector pDEF202 was digested with restriction enzymes *KpnI* and *SpeI* and then subjected to agarose gel electrophoresis to isolate a fragment containing mouse DHFR mini gene and SV40 polyadenylation signal, and the expression vector pDEF202-ghTPO was obtained by ligating, using T4 DNA ligase (manufactured by Takara Shuzo), the thus obtained fragment with a vector DNA which has been prepared by removing an SV40 polyadenylation signal-containing region

from a plasmid pEFHGTE by its treatment with restriction enzymes Kpn! and Spe!. This plasmid contains SV40 replication origin, human elongation factor 1- $\alpha$  promoter, SV40 early polyadenylation region for transcription of human TPO gene, mouse DHFR mini gene, pUC 18 replication origin and  $\beta$ -lactamase gene (Amp<sup>r</sup>), and human TPO chromosomal DNA is connected to a downstream site of the human elongation factor 1- $\alpha$  promoter.

## <Example 51>

## Expression of human TPO chromosomal DNA in CHO cells

CHO cells (a dhfr strain; Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, vol.77, p.4216, 1980) were grown by culturing them in an  $\alpha$ -minimum essential medium ( $\alpha$ -MEM(-), supplemented with thymidine and hypoxanthine) containing 10% fetal bovine serum using a plate of 6 cm in diameter (manufactured by Falcon), and the resulting cells were subjected to transfection by means of a calcium phosphate method (CellPhect, manufactured by Pharmacia).

That is, 10  $\mu g$  of the plasmid pDEF202-ghTPO prepared in Example 50 was mixed with 120  $\mu l$  of buffer A and 120  $\mu l$  of H<sub>2</sub>O, and the resulting mixture was incubated at room temperature for 10 minutes. This solution was further mixed with 120  $\mu l$  of buffer B and incubated at room temperature for additional 30 minutes. The thus prepared DNA solution was put in the plate and cultured for 6 hours in a CO<sub>2</sub> incubator. After removing the medium and washing the resulting plate twice with  $\alpha$ -MEM(-),  $\alpha$ -MEM(-) containing 10% dimethyl sulfoxide was added to the plate and treated for 2 minutes at room temperature. Next, non-selection medium ( $\alpha$ -MEM(-) op. cit., supplemented with hypoxanthine and thymidine) containing 10% dialyzed fetal bovine serum was added and cultured for 2 days, and then selection was carried out using a

selection medium ( $\alpha$ -MEM(-), free from hypoxanthine and thymidine) containing 10% dialyzed fetal bovine serum. Selection was carried out by treating the cells with trypsin, dispensing the treated cells in one plate of 6 cm in diameter into 5 plates of 10 cm in diameter or 20 plates of 24 well plates and continuing culturing of the cells while changing the selection medium every 2 days. The presence of human TPO activity was confirmed in the culture supernatant in the plates or wells in which proliferation of the cells was observed, when the human TPO activity was measured by the Ba/F3 assay.

In this instance, transfection of CHO cells may also be effected by carrying out co-transfection of CHO cells with pEFHGTE and pMG1.

### <Example 52>

Construction of vector for use in the expression in *E. coli* of human TPO protein (amino acid residues of positions 1 to 163) (to be referred to as "hMKT(1-163)" hereinafter) in which a Lys residue and a Met residue were respectively added to the -1 and -2 positions, and confirmation of its expression

In order to effect expression of a protein whose 1-and 3-position amino acid residues are the same as those of human TPO protein (amino acid residues of positions 1 to 163), a vector pCFM536/hMKT(1-163), also referred to as [Met-2, Lys-1]TPO(1-163), was constructed for use in the expression of hMKT(1-163) in *E. coli*, in the same manner as described in Example 42 using the following synthetic oligonucleotides 1-13, 2-13, 3-3 and 4-3:

1-13: 5'-CTAGAAAAACCAAGGAGGTAATAAATAATGAAATCTCCTGCACCA-3'

(SEQ ID NO:125);

2-13: 5'-CAGGTGGTGCAGGAGATTTCATTATTTATTACCTCCTTGGTTTTTT-3'

(SEQ ID NO:126);

3-3: 5'-CCTGCATGTGATTTACGGGTCCTGTCTAAACTGCTGCG-3'

(SEQ ID NO:127);

4-3: 5'-CGCAGCAGTTTAGACAGGACCCGTAAATCACATG-3

(SEQ ID NO:128);

10 1-13 20 30 40 CTAGAAAAAA CCAAGGAGGT AATAAATAAT GAAATCTCCT

TTTTTT GGTTCCTCCA TTATTTATTA CTTTAGAGGA

XbaI 2-13

50 60 <u>3-3</u> 70 80

GCACCACCTG CATGTGATTT ACGGGTCCTG TCTAAACTGC TGCG CGTGGTGGAC GTACACTAAA TGCCCAGGAC AGATTTGACG ACGC

<u>4-3</u>

(SEQ ID NO:129)

This expression plasmid contains a DNA sequence shown in the Sequence Listing (SEQ ID NO:12). Thereafter, expression of hMKT(1-163) was induced in the same manner as described in Example 43.

When the thus obtained expression protein was subjected to SDS-PAGE, transferred on a PVDF membrane and then subjected to N-terminal amino acid sequence analysis, it was confirmed that this protein contains the amino acid sequence of hMKT(1-163) to be expressed.

<Example 53>
Refolding of human TPO, h6T(1-163), derived from human TPO expressed in E. coli, using quanidine hydrochloride and glutathione, and purification and confirmation of its biological activity

A 1.2 g portion of frozen cells of the h6T(1-163)producing recombinant strain prepared in Example 43 were suspended in 3 ml of water and disrupted using a high pressure disintegrator. By subjecting the suspension to centrifugation, the precipitated fraction was recovered and most parts of contaminated proteins, cell components and the like were removed. The thus recovered precipitated fraction containing h6T(1-163) was suspended in 4 ml in final volume of water. A 1 ml portion of 1 M Tris buffer (pH 8.5) was added to the suspension with stirring, followed by further addition of 20 ml of 8 M guanidine hydrochloride and subsequent 5 minutes of stirring at room temperature to solubilize the contents. resulting solution was diluted 10 times with 20 mM Tris buffer (pH 8.5), 5 mM of reduced type glutathione and 0.5 mM of oxidized type glutathione were dissolved in the diluted solution with stirring and then the thus prepared solution was incubated overnight at 4°c. By centrifugation, h6T(1-163) was recovered in the supernatant fluid. A 160 ml portion of the thus obtained supernatant fluid was concentrated using YM10 ultrafiltration membrane (manufactured by Amicon) and subjected to buffer exchange with DPBS to obtain 3.4 ml in final volume of a fraction (protein concentration, 2.18 mg/ml). When this fraction was thoroughly dialyzed against IMDM culture medium and evaluated by the rat CFU-MK assay system, strong TPO activity (relative activity, 58,000) was found.

The TPO active fraction prepared above was subjected to an *in vivo* assay. That is, the active fraction was subcutaneously administered to ICR male mice (7 weeks of age), each group including 4 animals, daily for 5 consecutive days with a dose of 170  $\mu l$  (having a total activity 22,000 measured by the rat CFU-MK assay system) per animal. As a control, 170  $\mu l$  of DPBS was subcutaneously administered to each of 6 animals of the control group in the same manner. Just before the administration and on the next day of and 3 days after the completion of the administration, blood was collected from the eyeground to measure platelet counts using a microcell counter (F800, manufactured by Toa lyo Denshi). In

the TPO-administered group, platelet counts increased by a factor of about 2.15 in average on the next day of the completion of the administration in comparison with the value prior to the administration, and such an effect remained unchanged even after 3 days of the completion of the administration showing a 2.13 times higher value. Platelet counts on the next day of and 3 days after the completion of the administration was 1.73 and 1.80 times higher in TPO-treated group than in the control group with a significant difference (p <0.001, Student t-test) between them. These results demonstrated that the human TPO produced by *E. coli* and prepared by the above method has the ability to increase platelet counts in vivo.

Example 54>
Refolding of human TPO, h6T(1-163) expressed in E. colinusing sodium N-lauroyl sarcosinate and copper sulfate, and purification and confirmation of its biological activity

A 0.6 g portion of frozen cells of the h6T(1-163)producing recombinant strain prepared in Example 43 were suspended in 3 ml of water, disrupted using a high pressure disintegrator and then subjected to centrifugation to recover the precipitated fraction. After suspending the precipitated fraction in 3.1 ml of water, 0.19 ml of 1 M Tris buffer (pH 9.2), 11.25  $\mu l$  of 1 M DTT, 38  $\mu l$  of 0.5 M EDTA and 0.38 ml of 10% sodium deoxycholate solution were added to the suspension with stirring, and the resulting mixture was stirred at room temperature for 40 minutes. This was then subjected to centrifugation to recover the precipitated fraction and remove most parts of contaminated proteins, cell components and the like. The resulting precipitate was suspended in 4 ml of water and subjected to centrifugation to recover the precipitated The thus recovered fraction containing h6T(1-163). precipitated fraction was suspended in 3.8 ml of water, and

0.2 ml of 1 M Tris buffer (pH 8) and 1 ml of 10% sodium Nlauroyl sarcosinate were added to the suspension with stirring, followed by 20 minutes of stirring at room temperature to solubilize the contents. The resulting solution was mixed with 5 µl of 1% copper sulfate and stirred overnight (about 20 hours) at room temperature. The resulting solution was subjected to centrifugation to recover the supernatant fraction containing h6T(1-163), and a 5 ml portion of the supernatant was mixed with 5 ml of water and 10 ml of 20 mM Tris buffer (pH 7.7) and then with 2.6 g of 20 to 50 mesh Dowex 1-x4 chloride form ion exchange resin, followed by 90 minutes of stirring. The ion exchange resin was removed using a glass filter to recover the resin-nonadsorbed fraction which was subsequently subjected to centrifugation to recover the supernatant fluid. The thus obtained supernatant fluid was applied to an SP Sepharose Fast Flow cation exchange column which has been equilibrated in advance with 20 mM Tris buffer (pH 7.7) and eluted with the same buffer by a linear gradient of from 0 to 500 mM NaCl. When each of the eluted fractions of the SP Sepharose Fast Flow cation exchange column chromatography was thoroughly dialyzed against IMDM culture medium and evaluated by the rat CFU-MK assay system, strong TPO activity (relative activity, 19,000,000) was found in a fraction eluted with a NaCl concentration of about 100 mM. This fraction was concentrated 1.6 fold (2.5 ml; protein concentration, 25 µg/ml) using an ultrafiltration unit (Ultra Free CL, nominal molecular cutoff of 5,000, manufactured by Millipore, catalog No. UFC4LCC25). When the thus concentrated fraction was analyzed by SDS-PAGE in the presence of a reducing agent, a band corresponding to TPO was detected as the main band (purity, 70 to 80%).

The TPO active fraction prepared by the above procedure was examined in an *in vivo* assay. That is, the active fraction was subcutaneously administered to ICR male

mice (8 weeks of age), each group including 4 animals, daily for 5 consecutive days at a dose of 100  $\mu$ l (having a total activity of 47,500 measured by the rat CFU-MK assay system) per animal. As a control, 100 µl of 20 mM Tris buffer (pH 7.7) containing 100 mM NaCl was subcutaneously administered in the same manner. Just before the administration and on the next day of the completion of the administration, blood samples were collected from the eyeground to measure platelet counts using a microcell counter (F800, manufactured by Toa Iyo Denshi). In the TPO-administered group, platelet counts increased by a factor of about 1.73 in average after completion of the administration, in comparison with the number prior to the administration and were 1.59 times higher than in the control group with a significant difference (p <0.01, Student t-test) between them. These results demonstrated that the human TPO produced by E. coli and prepared by the above procedure has the ability to increase platelet counts in vivo.

### <Example 55>

#### Large scale culture of CHO cells

The large scale cultivation of the human TPO producing CHO cell line (CHO 28-30 cell, resistant to 25 nM MTX) which had been obtained by transfecting the human TPO expression plasmid pDEF202-hTPO-P1 into CHO cells in Example 32, was carried out as follows. The CHO cell line was cultured and proliferated in DMEM/F-12 culture medium (GIBCO) containing 25 nM MTX and 10% FCS. After the cells were detached with trypsin solution, 1 X 10<sup>7</sup> cells were inoculated in a roller bottle (ex Falcon, Falcon 3000) containing 200 ml of the same medium therein and then cultured at 37°c at a rolling speed of 1 rpm for 3 days. After 3 days culture, the culture supernatant was removed by suction and the adherent CHO cells were then rinsed with 100

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ml of PBS. 200 ml of the DMEM/F-12 medium (GIBCO) containing neither 25 nM MTX nor 10% FCS was added to the bottle and the cells were cultured at 37°c at a rolling speed of 1 rpm for 7 days. After 7 days culture, the culture supernatant was recovered as a starting material for subsequent purification procedure. The above mentioned procedures were carried out in 500 roller bottles to obtain 100 L of the culture supernatant.

## <Example 56>

## Purification of human TPO from human TPO producing CHO cell line

About 100 L of the serum free culture (1) supernatant obtained in Example 55 was filtered through a  $0.22~\mu m$  filter to obtain its filtrate which was then concentrated on an ultrafiltration unit (PLTK Pellicon cassette, nominal molecular weight 30,000 cutoff, ex After the replacement of the solvent of the Millipore). concentrate with pure water, p-APMSF (Wako Pure Chemicals) and Pefabloc SC (Merck), which are proteinase inhibitors, were added to the resulting aqueous solution at final concentrations of 1 mM and 0.35 mM respectively to obtain 3628 ml of the fraction of molecular weight 30,000 or greater (protein concentration: 1.60 mg/ml; total protein: 5805 mg; relative activity; 1,230,000; total activity: 7,149,000,000). fraction was subsequently subjected to the Western blot analysis as described in Example 45. As a result, the presence of TPO protein was revealed at a molecular weight ranging between 66,000 and 100,000. In examining in more detail, it was found that lower molecular weight TPO species than that of the above TPO were contained in the flow-through fraction.

The ultrafiltrate that contained the TPO with a molecular weight of not more than 30,000 was separately concentrated using an ultrafiltration unit (PLGC Pellicon

cassette, nominal molecular weight 10,000 cutoff, Millipore), to obtain 1901 ml of the low molecular weight TPO fraction (protein concentration: 0.36 mg/ml; total protein: 684 mg; relative activity: 245,500; total activity: 167,900,000). This reveals that the low molecular weight TPO species were generated during cell culture.

Next, to 3614 ml of the fraction containing the TPO having a molecular weight of 30,000 or greater were added 764 g of ammonium sulfate and 144.5 ml of 0.5 M sodium citrate buffer (pH 5.5) to make 4089 ml of the solution containing 1.41 M (final concentration) ammonium sulfate and 17.7 mM (final concentration) sodium citrate buffer. After insoluble substances were removed from the solution by centrifugation, the clear solution was obtained.

The clear solution was then applied to Macro-Prep Methyl HIC column (Bio-Rad, Catalog No. 156-0080; diameter 5 cm, bed height 24.5 cm) which had been previously equilibrated with 20 mM sodium citrate buffer (pH 5.5) containing 1.2 M ammonium sulfate at a flow rate of 25 ml/min. After the completion thereof, the elution was carried out with 20 mM sodium citrate buffer (pH 5.5) containing 1.2 M ammonium sulfate. The eluted fraction was subsequently concentrated on an ultrafiltration unit (ex Filtron, Omega Ultrasette, nominal molecular weight 8,000 cutoff) to obtain a flow-through fraction F1 (4455 ml; protein concentration: 0.400 mg/ml; total protein: 1780 mg; relative activity: 1,831,000).

Next, 20 mM Na citrate (pH 6.0) as an eluting buffer was passed through the column to obtain an eluate which was then concentrated using the same ultrafiltration unit (i.e., Omega Ultrasette, nominal molecular weight 8,000 cutoff) to collect a fraction F2 (1457 ml; protein concentration: 0.969 mg/ml; total protein: 1411 mg; relative activity: 1,715,000). In the F2 the presence of a protein having a molecular weight from 66,000 to 100,000 was confirmed on

SDS-PAGE. As a result of Western analysis, it was revealed that the protein was TPO.

Next, the F2 eluted from the Macro-Prep Methyl HIC column (1443 ml) was applied to SP Sepharose Fast Flow (Pharmacia Biotech, Catalog No. 17-0729-01; diameter 5 cm, bed height 12 cm) which has been previously equilibrated with 20 mM sodium citrate buffer (pH 6.0) at a flow rate of 15 ml/min. After the completion thereof, the elution was carried out with 20 mM sodium citrate buffer (pH 6.0) containing 50 mM NaCl. The eluate was collected and named fraction F1 (3.007 ml; protein concentration: 0.226 mg/ml; total protein 679 mg; relative activity: 88,830). Thereafter, the eluting buffer was replaced with 20 mM sodium citrate buffer (pH 5.4) containing 750 mM NaCl to collect fraction F2 eluted (931 ml; protein concentration: 0.763 mg/ml; total protein: 710 mg; relative activity: 5,558,000) which was then concentrated to 202 ml using ultrafiltration units (Filtron, Omega Ultrasette, nominal molecular weight 8,000 cutoff; and Amicon, YM 3 membrane).

Next, the concentrated TPO activity-containing fraction F2 (197 ml) was applied to a Sephacryl S-200HR gel filtration column (Pharmacia Biotech, Catalog No. 17-0584-05; diameter 7.5 cm, bed height 100 cm) at a flow rate of 3 ml/min. Elution volumes 1,200-1,785 ml, 1,785-2,010 ml, 2,010-2,280 ml and 2,280-3,000 ml were separately collected to obtain fractions F1 (585 ml; protein concentration: 1.00 mg/ml, total protein: 589 mg; relative activity: 4,118,000), F2 (225 ml; protein concentration: 0.263 mg/ml; total protein: 59.2 mg; relative activity 2,509,000), F3 (270 ml; protein concentration: 0.119 mg/ml, total protein; 32.1 mg; relative activity: 2,535,000) and F4 (720 ml; protein concentration: 0.0467 mg/ml; total protein: 33.6 mg; relative activity: 1,155,000), respectively. Thus, the fractionated TPO activity had a broad molecular weight range as determined by gel

filtration. After SDS-PAGE of the fractions followed by Western blot analysis, it was found that F1 had a TPO molecule species with a molecular weight of 66,000 to 1,000,000 predominantly; F2 a TPO molecule species with a molecular weight of 32,000 to 60,000; and F3 a TPO species with a molecular weight of 32,000 to 42,000. All the TPO molecules possessed TPO activity.

On the basis of the result of N-terminal amino acid sequencing of the TPO molecule having a molecular weight of 66,000 to 100,000, we confirmed that the TPO molecule had the amino acid sequence of a human TPO gene encoded protein.

In addition, the TPO molecule having a molecular weight 66,000-100,000 was subjected to enzymatic digestion experiments using the glycosidase enzymes consisting of N-glycanase (ex Genzyme, Catalog No. 1472-00), neuraminidase (Nakarai-Tesqu, Catalog No. 242-29SP), endo-a-N-acetylgalactosaminidase (Seikagaku Kogyo, Catalog No. 100453) and O-glycosidase (Boehringer Mannheim Biochemica, Catalog No. 1347101) singly or in combination, and then to SDS-PAGE analysis. As a result, the polypeptide portion of the TPO was found to have a molecular weight of about 36,000 as expected from its theoretical molecular weight and to be a glycoprotein with both N- and O-linked sugar chains.

#### <Example 57>

## Purification of human TPO from human TPO producing CHO cell line

(1) To 1 L of the serum free culture supernatant of the CHO cells obtained in the same manner as in Example 55 was added 211.4 g of ammonium sulfate, after which the mixture was filtered through a 0.2 μm filter (ex Gelman Science, Catalog No. 12992). The obtained filtrate was applied to Macro-Prep Methyl HIC column (Bio-Rad, Catalog No. 156-0081, diameter 50 mm, bed height 90 mm) which had been

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previously equilibrated with 20 mM sodium acetate buffer (pH 5.6) containing 1.2 M ammonium sulfate at a flow rate of 15 ml/min. After the completion thereof, the elution was carried out with 450 ml of 20 mM sodium acetate buffer (pH 5.6) containing 1.2 M ammonium sulfate. The eluted fraction was then applied to a reverse phase Vydac C4 column (The Separations Group, Catalog No. 214BTP54, diameter 4.6 mm, bed height 250 mm) at a flow rate of 0.75 ml/min. After the column was washed with 10 mM Tris buffer (pH 6.4) containing 5% ethanol (referred to as "development solvent A") for 15 min, the elution was carried out with a 66-min linear gradient of from the development solvent A to 10 mM Tris buffer (pH 6.4) containing 94% ethanol (referred to as "development solvent B"). The chromatogram obtained is shown in Fig. 15. As a result of SDS-PAGE analysis, the molecule having a molecular weight 65,000-100,000 which was expected to be TPO and which corresponded to the fraction of retention time 68-72 min after addition of the sample, appeared as a single band on the SDS-PAGE gel (see Fig. 16). Western analysis further revealed that the obtained protein is TPO.

The N-terminal amino acid analysis of the protein sample revealed that the protein had the amino acid sequence of a human TPO gene encoded protein.

<Example 58>
Preparation of recombinant virus for
human TPO-expression within insect cells

The plasmid pHTP1 prepared in Example 30 was digested with restriction enzymes EcoRI and NotI and then subjected to 1% agarose gel electrophoresis to obtain an about 1200-bp band which was subsequently purified using Prep-A-Gene DNA purification kit. The purified DNA was then ligated to a transfer vector pVL 1393 (Invitrogen) pretreated with the same restriction enzymes, followed by transformation into

Competent-High E. coli DH5 (Toyo Boseki). From the colonies obtained was selected a clone pVL1393/hTPO which contained a full length coding region of human TPO cDNA. The plasmid DNA was then prepared from the clone by the method described in Molecular Cloning (Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) followed by transfection into insect cell Sf 21 (Invitrogen) using BaculoGold<sup>TM</sup> Transfection kit (Farmingen), and the transfected cells were cultured in Sf-900 medium ( Lifetechnology) at 27°c for 4 days to recover the supernatant containing the virus. The supernatant was then diluted to  $1:10^9 \sim 1:10^5$ , and about 7 X  $10^5$  Sf 21 cells were infected with 1 ml of the diluted supernatant at 27°c for 1 hr in a 35-mm (in diameter) shale. After removal of the supernatant, 1% hot agarose in Sf-900 medium was placed into the culture and allowed to solidify, followed by 6-day cultivation at 27°c in a moisturized atmosphere. A single plaque formed was picked up, from which the virus was released in 200 µl of Sf-900 medium. Sf 21 cells were infected with the obtained viral clone in a 24-well plate to proliferate the virus. A portion of the virus-containing liquid from the single plaque was treated with phenol/chloroform followed by ethanol precipitation to recover the viral DNA which was then used as a template to carry out PCR using primers specific for human TPO cDNA. The recombinant virus containing human TPO cDNA was selected based on amplification of the specific DNA fragment. Then, Sf 21 cells were infected with the supernatant containing the recombinant virus carrying human TPO cDNA to proliferate the virus.

<Example 59>
Expression of human TPO in Sf 21 insect
cells and identification of TPO activity

Sf 21 cells were cultured in a 175 cm<sup>2</sup> culture flask to about 80% confluence was attained, followed by

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infection with the human TPO cDNA carrying recombinant virus prepared in Example 58 at 27°c for 1 hr, after which the obtained cells were cultured in Sf-900 medium at 27°c for 4 days to recover the supernatant of the culture. The obtained culture supernatant was substituted by IMDM culture medium on NAPTM-5 column (Pharmacia). Significant TPO activity in the resultant supernatants was detected in a dose-dependent manner in both rat CFU-MK assay and M-07e assay. Also, the recombinant human TPO expressed in Sf 21 cells was identified by means of the Western analysis as described in Example 45.

<Example 60>

Refolding of variant human TPO, h6T(1-163) derived from clone pCFM536/h6T(1-163) carrying a human TPO base sequence via its expression in E. coli. by using sodium N-lauroyl sarcosinate and cupric sulfate, and purification of the h6T(1-163)

Thirty g of the frozen h6T(1-163)-producing recombinant microorganism prepared in Example 43 was suspended in 300 ml of water, disrupted in a high pressure disrupting apparatus (10,000 psi, Rannie High Pressure Laboratory), and then centrifuged to recover a sedimented fraction. The sediment fraction was suspended in 90 ml of water to which water was then added to the total amount of 150 ml with stirring. Subsequently, to the mixture was added 9 ml of 1 M Tris buffer (pH 9.2), 540 µl of 1 M DTT, 1.8 ml of 0.5 M EDTA and 18 ml of 10% sodium deoxycholic acid with stirring, followed by 30 min-stirring at room temperature. The sedimented fraction was recovered by centrifugation and most of the contaminated proteins and microorganism components were removed. To the sediment was added 180 ml of 5 mM DDT to obtain a suspension which was then centrifuged to recover the sediment fraction containing h6T(1-

163). To the resultant sediment was added 300 ml of water to obtain a suspension to which water was then added to the liquid amount of 570 ml with stirring. Thirty ml of 1 M Tris buffer (pH 8), 150 ml of 10% sodium N-lauroyl sarcosine were added to the mixture at room temperature with 20-min stirring in order to solubilize the h6T(1-163). To this was further added 750 µl of 1% cupric sulfate and the mixture was stirred overnight (about 20 hr) at room temperature. After the supernatant fraction containing h6T(1-163) was recovered by centrifugation, to 750 ml of the supernatant was added 750 ml of water and 1,500 ml of 20 mM Tris buffer (ph 7.7) followed by addition of 3 ml of polysorbate 80 (Nikko Chemicals) with stirring. To the obtained solution was added 600 g of Dowex 1-X4 (20-50 mesh, chloride form) ion-exchange resin followed by 90-min stirring at room temperature. Following recovery of the non-adsorbed fraction using a glass filter, the ionexchange resin was washed with 750 ml of 20 mM Tris buffer (pH 7.7). The combined solution of the non-adsorbed and wash fraction was adjusted to pH 9.2 with 2 N sodium hydroxide after which it was applied to Q Sepharose Fast Flow anionexchange column (ID 5 cm X 10 cm) equilibrated with 20 mM Tris buffer (pH 9.2) containing 0.1% polysorbate 80 to recover a non-adsorbed fraction. The pH of the obtained non-adsorbed fraction was adjusted to 7.2 with hydrochloric acid, applied to SP Sepharose Fast Flow cation exchange column (ID 5 cm X 10 cm) equilibrated with 20 mM Tris buffer (pH 7.2) containing 0.1% polysorbate 80, and then eluted with the linear gradient from 0 M to 500 mM NaCl in the same buffer. fractions were subjected to SDS-PAGE analysis to collect a TPO fraction to which trifluoroacetic acid was then added to a concentration of 0.1%. After the solution obtained was applied to Capcell Pak 5 µm 300 A C1 column (ID 2.1 cm X 5 cm X 2, Shiseido), a reverse phase HPLC was carried out by the linear gradient elution method with increasing 1-propanol concentration in 0.1% trifluoroacetic acid. The eluate was analyzed on SDS-PAGE in the absence of a reducing agent. As a result, three fractions were fractionated based upon the elution position in the reverse phase HPLC, each fraction of which was diluted three times with 0.1% trifluoro-acetic acid. Each fraction diluted was applied to the above reverse phase HPLC column in the similar manner. The resultant three TPO fractions which were designated as Fr. S-a, Fr. S-b and Fr. S-c depending in the order of elution on the reverse phase HPLC (see Fig. 17), were analyzed on SDS-PAGE in the absence of a reducing agent. As a result, a single band was detected at a molecular weight of about 18 kDa (Fr. S-a), about 19 kDa (Fr. S-b) or 18 kDa (Fr. S-c) (see Fig. 18). Following their amino acid analyses, each of the amino acid compositions determined was almost consistent with the corresponding theoretical value estimated from the sequence information. Additionally, the results of N-terminal amino acid analysis showed that they were the expected sequences. The protein amount of each fraction determined by amino acid analysis was 0.64 mg (Fr. Sa), 1.81 mg (Fr. S-b) or 3.49 mg (Fr. S-c). After full dialysis of each fraction against IMDM medium, M-07e assay was carried As a result, the TPO relative activity of each fraction was about 1,620,000 (Fr. s-a), 23,500,000 (Fr. S-b) or 746,000,000 (Fr. S-c).

Example 61>
Refolding of variant human TPO. h6T(1-163) derived from clone pCFM536/h6T carryinga human TPO base sequence via its expression in E. coli. by using guanidine hydrochloride and cystein-cystin. and purification of the h6T(1-163).

Fifty g of the h6T(1-163)-producing frozen recombinant microorganism prepared in Example 43 was added to 500 ml of water and suspended, disrupted using a high

pressure disrupting apparatus (10,000 psi, Rannie High Pressure Laboratory), and then centrifuged to recover a The sediment fraction was suspended in water and its liquid amount was then adjusted to 250 ml by addition of water with stirring. To this, thereafter, 15 ml of 1 M Tris buffer (pH 9.2), 900  $\mu l$  of 1 M DTT, 3 ml of 0.5 M EDTA and 30 ml of 10% sodium deoxycholic acid were added and stirred at room temperature for 30 min: After centrifugation, the sediment fraction was recovered while removing most of the contaminated proteins, microorganism components and the like. To the sediment was added 300 ml of 5 mM DTT and suspended, after which the sedimented fraction containing h6T(1-163) was recovered by means of centrifugation. recovered sedimented fraction containing h6T(1-163) was suspended in water in a total volume of 104 ml. suspension was added 20 ml of 1M Tris buffer (pH 8.5) and then 376 ml of 8 M guanidine hydrochloride with stirring, and subsequently stirred at room temperature for 10 min in order to solubilize the h6T (1-163). To this was added 2,500 ml of 20 mM Tris buffer (pH 8.5) containing 0.1% polysorbate 80 and then 2,000 ml of 20 mM Tris buffer (pH 8.5) containing 1 M. guanidine hydrochloride with stirring, followed by addition of 5 mM cystein and 0.5 mM cystin. The thus obtained solution was incubated overnight at 4°c and then centrifuged to recover h6T(1-163) in the supernatant which was then concentrated using Prep Scale UF cartridge PLDC ultrafiltration membrane (Millipore). The buffer of the concentrate was replaced by 20 mM Tris buffer (ph 9.2) containing 0.1% polysorbate 80 until reaching a final volume of 1,000 ml. The obtained solution was applied to Q Sepharose Fast Flow anionic exchange column (ID 5 cm X 10 cm) equilibrated with 20 mM Tris buffer (pH 9.2) containing 0.1% polysorbate 80, and then eluted with a linear gradient from 0 M to 500 mM NaCl in the same buffer, by which the fraction (240 ml) eluted at about 20 mM - about 150 mM

NaCl was recovered. The resultant fraction was diluted four times with 20 mM Tris buffer (pH 7.2) to attain a total volume of 960 ml, and then adjusted to pH 7.2 with acetic acid, applied to SP Sepharose Fast Flow cation exchange column (ID 5 cm X 10 cm) equilibrated with 20 mM Tris buffer (pH 7.2) containing 0.1% polysorbate 80, and then eluted with a linear gradient from 0 M to 500 mM NaCl in the same buffer. The eluate was analyzed on SDS-PAGE to collect a TPO fraction. To the TPO fraction was added trifluoroacetic acid until its final volume of 0.1%. The resultant solution was applied to Capcell Pak 5 µm 300A C1 column (ID 2.1 cm X 5 cm X 2, Shiseido), and reverse phase HPLC was subsequently conducted by linear method increasing gradient elution with 1-propanol concentration in 0.1% trifluoroacetic acid. The eluate was analyzed by SDS-PAGE in the absence of a reducing agent and fractionated into two fractions depending on the eluted position in the reverse phase HPLC. The two TPO fractions were designated as Fr. G-a and Fr. G-d based on their order eluted in the reverse phase HPLC (see Fig. 17). Each fraction was then analyzed on SDS-PAGE in the absence of a reducing agent. As a result, a single band was detected at a molecular weight of about 18 kDa (Fr. G-a) or about 32 kDa (Fr. G-d) (see Fig. 18). Furthermore, the SDS-PAGE analysis of the above fractions in the presence of a reducing agent showed that in both the fractions a single band was detected at a molecular weight of about 20 kDa. The result of the amino acid analysis of the fractions showed that each of the amino acid compositions thereof was almost consistent with the corresponding theoretical value estimated from the sequence information. Additionally, the results of the N-terminal amino acid analysis showed that they were the expected sequences. The protein amount of each fraction determined from the results of the amino acid analysis was 2.56 mg (Fr. G-a) or 1.16 mg (Fr. G-d). Following full dialysis of each fraction against IMDM medium, M-07e assay was carried out. As a result, the fraction had TPO relative activity of about 3,960,000 (Fr. G-a) or of about 7,760,000 (Fr. G-d).

<Example 62>

Construction of recombinant vector pDEF202-hTPO163 for expression of a partial length human TPO (amino acids 1 to 163) (hereinafter referred to as "hTPO163") within CHO cells

The vector pDEF202 constructed in Example 31 was treated with restriction enzymes EcoRI and SpeI followed by recovery of a larger vector fragment using agarose gel electrophoresis. The fragment was ligated by T4DNA ligase (Takara-Shuzo) with an hTPO163 cDNA which had been prepared by treating a plasmid pEF18S-hTPO163 containing the hTPO163 cDNA encoding the amino acids -21 to 163 (SEQ ID NO:13) with restriction enzymes EcoRI and SpeI, to give an expression vector pDEF202-hTPO163. This plasmid contained an origin of replication of SV40, a human elongation factor 1- $\alpha$ -promoter, an SV40 early polyadenylation site, a murine DHFR minigene, an origin of replication of pUC18 and a  $\beta$ -lactamase gene (Ampr), in which the hTPO163 cDNA is linked at a site downstream of the human elongation factor 1- $\alpha$ -promoter.

#### <Example 63>

# Expression of hTPO163 in CHO cells

A CHO cell line (dhfr-strain, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA,  $\overline{ZZ}$ , p 4216, 1980) was grown in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM(-) with thymidine and hypoxanthine) containing 10% bovine fetal serum in 6 cm dish (Falcon), and then transformed with a pDEF202-hTPO163 plasmid by the transfectum method (Seikagaku Kogyo K.K.).

Briefly, 10  $\mu g$  of the plasmid pDEF202-hTPO163 prepared in Example 62 was mixed with 240  $\mu l$  of 0.3 M NaCl then with a mixture of 20  $\mu l$  of transfectum and 220  $\mu l$  of  $H_2O$ .

This DNA solution was added dropwise to the dish followed by culture for 6 hr in a CO2 incubator. The medium was removed from the dish which was then washed twice with  $\alpha\text{-MEM}(-)$ followed by addition of 10% DMSO containing α-MEM(-) before incubation for 2 minutes at room temperature. Thereafter, to the dish was added 10% dialyzed bovine fetal serum-containing non-selection medium ( $\alpha$ -MEM(-) with hypoxanthine and thymidine) followed by two day culture, then selection in 10% dialized bovine fetal serum-containing selection medium ( $\alpha$ -MEM(-) without hypoxanthine and thymidine). The selection was conducted by trypsinizing the cells, dividing them into five 10 cm dishes or twenty 24 well dishes per the above 6 cm dish, and continuing to culture while the medium was exchanged with the fresh selection medium at intervals of two days. The supernatants from plates or wells were assayed for TPO activity using Ba/F3 assay, thereby the TPO activity being observed. The cells in which TPO activity was observed in the culture supernatants were then transferred to fresh plates or wells after divided to a cell concentration of 1:15 with the selection medium containing 25 nM methotrexate, and subsequently recultured for growth and cloning of cells resistant to methotrexate.

Alternatively, the transformation of the CHO cells can be carried out by co-transfection of pEF18S-hTPO163 and pMG1 into the CHO cells.

CHO strain (CHO-DUKXB11) transfected with the plasmid pDEF202-hTPO163 has been deposited by the present applicant on January 31, 1995, under the accession No. FERM BP-4989, at the National Institute of Bioscience and Human Technology, Agencey of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

<Example 64>
Large-scale culture of CHO cells

The large-scale culture of the hTPO163-producing CHO cell line (CHO 109 cells, obtained by the above-mentioned selection in 10% dialyzed bovine fetal serum-containing selection medium [ $\alpha$ -MEM $^{-}$  without hypoxanthine and thymidine]) which had been obtained by transfection of the hTPO163-expression plasmid pDEF202-hTPO163 into CHO cells in Example 63 was carried out as follows. The CHO 109 cells were grown in DMEM/F-12 medium (GIBCO) with 10% FCS. After the cells were harvested (or trypsinized) using a trypsin solution, ten millions cells were inoculated into a Falcon roller bottle (Falcon 3000) containing 200 ml of the same medium and then cultured at a rolling speed of 1 rpm at 37°C for 3 days. The culture medium was removed under suction and the surface of the cell culture was rinsed with 100 ml of PBS. Then, to the cell culture was added 200 ml of DMEM/F-12 medium (GIBCO) without 10% FCS followed by 7 day culture at 37°c at 1 rpm. The culture supernatant harvested was used as a starting material for the subsequent purification step. The similar procedures were conducted in 300 roller bottles to yield 60 L of the serum-free culture supernatant.

<Example 65>

Purification of hTPO163 from hTPO163-producing CHO cell line (1) 60 L of the serum-free culture supernatant obtained in Example 64 was filtered through a 0.22 μm filtration filter to collect a filtrate which was then concentrated using an ultrafiltration unit (Filtron, molecular weight 10,000 cutoff) to obtain a concentrated fraction (600 ml, 11.2 mg protein/ml, total protein 6430 mg). By Western analysis of this fraction using the anti-HT1 peptide antibody, the presence of hTPO163 protein expressed at the range of an apparent molecular weight from 20,000 to 26,000 was shown.

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The resulting concentrated culture supernatant (537 ml) was treated on a Sephadex G-25 Fine column (Pharmacia-Biotech, Catalogue No. 17-0032-02; diameter 10 cm and bed height 30 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.8) to obtain a protein fraction F1 (938 ml, protein concentration 4.9 mg/ml, total protein 4594 mg) as a solution in 10 mM sodium phosphate buffer (pH 6.8).

This protein fraction (929 ml) from Sephadex G-25 Fine column was applied at a flow rate of 15 ml/min, to a SP Sepharose Fast Flow column (Farmacia-Biotech, Catalogue No. 17-0729-01; diameter 5 cm and bed height 12 cm) preequilibrated with 10 mM sodium phosphate buffer (pH 6.8) followed by elution with 10 mM sodium phosphate buffer (pH 6.8) then the same buffer containing 10% ethanol. The eluates were combined as a fraction F1 (1608 ml, protein concentration 2.13 mg/ml, total protein 3426 mg). Then, the second elution was carried out with 10 mM sodium phosphate buffer (pH 6.8) containing 750 mM NaCl and 25% ethanol to collect a main hTPO163 eluate of fraction F2 (651 ml, protein concentration 1.67 mg/ml, total protein 1087 mg).

To the TPO activity-containing fraction F2 (200 ml) from SP Sepharose Fast Flow column were added ethanol and purified water to prepare 300 ml of 45% (final conc.) ethanol solution. Insoluble materials which resulted from the addition of ethanol were removed by centrifugation. The supernatant was injected into a SOURCE 15RPC column (Pharmacia-Biotech, Catalogue No. 17-0727-02; diameter 2 cm and bed height 20 cm) pre-equilibrated with 50% of solvent A (10 mM sodium acetate buffer, pH 6.7) plus 50% of solvent B (10 mM sodium acetate buffer, pH 6.7, containing 90% ethanol) at a flow rate of 2 ml/min. Then the column was washed with 55% solvent A plus 45% solvent B until unadsorbed substances were almost completely eluted, after which the elution at a flow rate of 1.5 ml/min was carried out according to the

following elution profile: 50% B for 5 minutes; linear gradient from 50% B to 100% B over 140 minutes; and then 100% B for 35 minutes. The fractions were collected at intervals of 5 minutes (corresponding to 7.5 ml volume). All the fractions were subjected to SDS-PAGE then to Western analysis for examining an elution range of hTPO163. As a result, the highly purified hTPO163 with an apparent molecular weight from approximately 20,000 to 26,000 was found to be eluted at the range from 66% to 87% ethanol. Among these hTPO163 proteins, the hTPO163 having a higher molecular weight was eluted earlier from the reverse phase column, suggesting that the glycosylated hTPO163 molecule has an increased hydrophilicity.

To 88.8 ml of the hTPO163 elution fraction (i.e. hTPO163 fraction (90 ml) eluted at the range from 68% to 86.5% ethanol) from SOURCE 15RPC column was added CHAPS, and the mixture was concentrated and washed to obtain 2.5 ml of a concentrate containing about 5% ethanol and 4 mM CHAPS. This concentrate was subsequently applied to a Superdex 75 pg column (Pharmacia-Biotech, Catalogue No. 17-1070-01; diameter 2.6 cm and bed height 60 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 10% ethanol at a flow rate of 1.5 ml/min. From the time of 60 minutes after application, the elution fractions were collected in an amount of 6 ml each (i.e. every 4 min). As a result, the hTPO163 was eluted in the fractions from tube number 16 to at least tube number 31 as determined by SDS-PAGE. This elution position is corresponding to the range of a molecular weight from approximately 44,000 to approximately 6,000, as determined by gel filtration using a standard molecular weight marker (mixture of Bio-Rad, Gel Filtration Standard, Catalogue No. 151-1901; and Calbiochem, insulin, Catalogue No. 407696). Additionally, these hTPO163 molecules appeared to be eluted in the order of decreasing degree of glycosylation. All the

hTPO163 species of the range from tube numbers 16 to 31 (elution volume: 180 to 276 ml) were collected as fraction FA. In addition to the fraction FA, the fractions of the tube Nos. 16 to 18 (elution volume: 180 to 198 ml), 19 to 24 (elution volume: 198 to 234 ml) and 25 to 31 (elution volume: 234 to 276 ml) were separately collected and designated as fractions FH, FM and FL, respectively. Also, the fraction FA was prepared by combination of the portions of the fractions FH, FM and FL. The above is shown in Fig. 19.

(2) Next, the hTPO163 elution fractions FH, FM, FL and FA from the Superdex 75 pg column set forth in (1) will be The N-terminal amino acid illustrated in more detail. sequences of the hTPO163 species obtained above were determined by the same method as in Example 1, but most of the N-terminal Ser residues could not be identified. suggests that an O-linked sugar is added to the N-terminal Ser. Furthermore, the sequence following the N-terminal Ser was confirmed to be an amino acid sequence expected from the gene sequence of hTPO. The concentrations of the hTPO163 proteins in the FH, FM, FL and FA were 10.2 ng/ml, 6.2 ng/ml, 0.84 ng/ml and 3.2 ng/ml as determined by amino acid analysis (AccQ. Tag method, Wasters), respectively, provided that the concentrations were of the peptide moieties not containing any sugar chain. These fractions (100 ng each) were subjected to SDS-PAGE under unreducing conditions using a multigel 15/25 (Dai-ichi Kagaku Yakuhin, 15 to 25% precast polyacrylamide gel) or under reducing conditions using DTT, followed by silver-staining (Daiichi Pure Chemicals). As a result, each of these fractions was found to contain highly pure hTPO163. The apparent molecular weights of the hTPO163 in the FH, FM, FL and FA, as calculated using DPCIII molecular weight marker (Daijchi Pure Chemicals) as a standard under the reducing conditions, were 24,000 to 21,500, 23,000 to 21,000, 23,000 to 20,500 and 23,500 to 20,500, respectively (see Fig. 20).

Additionally, the apparent molecular weights of the hTPO in the FH, FM, FL and FA, as calculated using biotin-labelled SDS-PAGE standard (Bio-Rad, Biotynylated SDS-PAGE Standards, Broad Range: Catalogue No. 161-0319) under the reducing conditions on Western analysis, were 26,000 to 22,000, 25,500 to 22,000, 26,000 to 21,000 and 26,000 to 21,000, respectively. The heterogeneity among the molecular weights of FH, FM, FL and FA may be due to the heterogeneity of the Olinked sugar chains. Therefore, each fraction of the FH, FM, FL and FA was digested with neuraminidase (Neuraminidase, Nacalai tesque, Catalogue No. 242-29SP), reduced with DTT. and then analyzed on SDS-PAGE. As a result, in all the fractions, the apparent molecular weight was around 19,000. revealing that the heterogeneity of the molecular weight of hTPO is mainly due to the heterogeneity of the amount of sialic acid in the sugar chains coupled with the hTPO163 protein and that the hTPO163 obtained was expressed as a glycoprotein in CHO cells.

(3) The fractions FH, FM, FL and FA obtained in (2) were assayed for their <u>in vitro</u> activity by M-07e assay system. As a result, the relative specific activity were 511,000,000, 775,000,000, 1,150,000,000 and 715,000,000/mg hTPO163 protein (weight of the peptide moiety not containing any sugar weight).

# <Example 66>

Construction of E. coli vector for expression of human TPO (amino acids 1 to 332) in which Lys is added at the position-1 and Met at the position-2 respectively (hereinafter referred to as "hMKT(1-332)"), and expression of the hMKT(1-332)

To express the full length amino acid sequence of human TPO in <u>E. coli</u>, the use codons of from amino acid 164 to amino acid 332 were changed to <u>E. coli</u> preference codons as described below.

# Table 5

21:5-CTCCCGAACCGTACCAGCGGCCTGCTGGAAACCAACTTTACCGCGAG-3	
	(SEQ ID NO:130)
22:5-GGTAAAGTTGGTTTCCAGCAGGCCGCTGGTACGGTTCGGGAGC	ाट्डा-उ
	(SEQ ID NO:131)
23:5-CGCGCGTACCACCGGCAGCGGCCTGCTGAAATGGCAGCAGGGC	गाट्डा ४
·	(SEQ ID NO:132)
24:5-AGCCCTGCCGCATTTCAGCAGGCCCCTGCCGGTGGTACGCGCC	
	(SEQ ID NO:133)
25:5-GCGAAAATCCCGGGCCTGCTGAACCAGACCAGCCGTAGCCTGG	
	(SEQ ID NO:134)
26:5-ATCCAGGCTACGGCTGGTCTGGTTCAGCAGGCCCGGGATTTTCC	
	(SEQ ID NO:135)
27:5-CCCGGGCTATCTGAACCGTATCCATGAACTGCTGAACGGCACCC	
	(SEQ ID NO:136)
28:5-GTGCCGTTCAGCAGTTCATGGATACGGTTCAGATAGCCCGGGA	
	(SEQ ID NO:137)
29:5-CCCTGTTTCCCGGCCCGAGCCGTCGCACCCTGGGCGCGCCGCAGA	(SEQ ID NO:138)
30:5-ATCCGGCGCCCCAGGGTGCGACGGCTCGGGCCCGGAAACAGG	•
30:5410033333333333333333333333333333333333	(SEQ ID NO:139)
31:5-ATCAGCTCTGGCACCAGCGATACCGGCAGCCTGCCGCCGAACC	•
31:5-410-430101630-400-450541-400530-4001000000000000000000000000000000000	(SEQ ID NO:140)
2:5-CAGGTTCGGCGGCAGGCTGCCGGTATCGCTGGTGCCAGAGCTG	•
2,50401100000010000111100010010110010010010	(SEQ ID NO:141)
33:5-GGGCTATAGCCCGAGCCCGACCCATCCGCCGACCGACCAGTAT	•
	(SEQ ID NO:142)
34:5-GGTATACTGGCCGGTCGGCGGATGGGTCGGGCTCGGGCTATAG	CCCGCTG-3
	(SEQ ID NO:143)
35:5-TCCGCTGCCGCCGACCCTGCCGACCCCGGTGGTTCAGCTGCATCCGCTGC3	
	(SEQ ID NO:144)
36:5-GGATGCAGCTGAACCACCGGGGTCGGCAGGGTCGGCGGCCAGCG	GAAACAG3
•	(SEQ ID NO:145)

37:5-TGCCGGATCCGAGCGCGCCGACCCGACCCGACCAGCCCGCTGCTGAACA3
(SEQ ID NO:146)

39:5-CCAGCTATACCCATAGCCAGAACCTGAGCCAGGAAGGCTAATGAAGCTTGA-3'
(SEQ ID NO:148)

40:5-CTTCATTAGCCTTCCTGGCTCAGGTTCTGGCTATGGGTATAGCTGGTGTTC-3' (SEQ ID NO:149)

41:5-ACGAGCTCCCGAACCGTACCA-3' (SEQ ID NO:150)
42:5-CTGATATCCGGCGCGCCCAGG-3' (SEQ ID NO:151)
43:5-CGGATATCAGCTCTGGCACCA-3' (SEQ ID NO:152)
44:5-TCAAGCTTCATTAGCCTTCCT-3' (SEQ ID NO:153)

The synthetic oligonucleotides: 21 and 22; 23 and 24; 25 and 26; 27 and 28; 29 and 30; 31 and 32; 33 and 34; 35 and 36; 37 and 38; or 39 and 40, were phosphorylated in a solution of 0.1 mM ATP, 10 mM Tris-acetate, 10 mM Mgacetate, 50 mM K-acetate using T4 kinase (Pharmacia) in a Then 1/10 volumes of a solution of 100 mM same tube. Tris/HCI (pH 7.5), 100 mM MgCl<sub>2</sub>, 500 mM NaCl was added to the reaction mixture, boiled for 3 minutes in a water bath, and allowed to cool to form a double stranded DNA. Four sets of the double stranded DNAs, i.e. oligonucleotides 21 and 22/23 and 24 (combination-A); oligonucleotides 25 and 26/27 and 28 (combination-B); oligonucleotides 31 and 32/33 and 34 (combination-C); and oligonucleotides 35 and 36/37 and 38/39 and 40 (combination-D), were separately ligated with a DNA ligation kit (Takara-Shuzo), and thereafter the combination-A was ligated together with the combination-B and similarly the combination-C with the combination-D to produce ligation-1 and ligation-2 respectively. Using the ligations-1 and -2 as templates, PCR were carried out wherein the oligonucleotides-41 and -42 for ligation-1, as well as the oligonucleotides-43 and -44 for ligation-2, were employed as primers.

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products from the PCR of the ligations-1 and -2 were digested with SacI and EcoRV and with EcoRV and HindIII respectively, followed by electrophoresis on 2% agorose gel and then purification using a Prep-A-Gene DNA Purification Kit to recover about 240-bp and 250-bp fragments respectively. The two fragments thus obtained were subcloned into pBluescript II KS+ (Stratagene) predigested with SacI and HindIII (E. coli DH5 was employed as a host.). Of the resulting colones, the clone having the base sequence shown in Table 6 was selected through sequencing and named pBL(SH)(174-332) (see SEQ ID NO:154).

#### Table 6

CTC CCG AAC CGT ACC AGC GGC CTG CTG GAA ACC AAC TTT ACC GCG AGC Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr Ala Ser 174 180 GCG CGT ACC ACC GGC AGC GGC CTG CTG AAA TGG CAG CAG GGC TTT CGT Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly Phe Arg 200 190 GCG AAA ATC CCG GGC CTG CTG AAC CAG ACC AGC CGT AGC CTG GAT CAG · Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln 220 210 ATC CCG GGC TAT CTG AAC CGT ATC CAT GAA CTG CTG AAC GGC ACC CGT Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly Thr Arg 230 GGC CTG TTT CCG GGC CCG AGC CGT CGC ACC CTG GGC GCG CCG GAT ATC Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro Asp Ile 240 AGC TCT GGC ACC AGC GAT ACC GGC AGC CTG CCG AAC CTG CAG CCG Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu Gln Pro 260 GGC TAT AGC CCG AGC CCG ACC CAT CCG CCG ACC GGC CAG TAT ACC CTG Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr Thr Leu 280 270

Next, the pBL(XH)h6T(1-163) prepared in Example 42 as a template was subjected to PCR in the presence of the following synthetic oligonucleotides 45 and 46 as primers, and the PCR product was digested with BamHI and SacI followed by electrophoresis on 6% polyacrylamide gel to recover an about 160-bp fragment from the gel.

45:5-AAGGATCCGAACGCTATCTTCCTG-3" (SEQ ID NO:155) 46:5-GGGAGCTCGTTCAGGGTCAGAACCAGA

GAGGTACGAGACGGAACAGCAGTGGTTGG-3'(SEQ ID NO:156)

Also, the pBL(SH)(174-332) was digested with Sacl and HindIII and the digest was then purified using the Prep-A-Gene DNA Purification Kit to give an about 480-bp fragment. The two fragments prepared above were subcloned into pBluescript II KS+ (Stratagene) predigested with BamHI and HindIII (E. coli DH5 was employed as a host.).

Of the resulting clones, the clone having the base sequence shown in Table 7 was selected through sequencing and named pBL(BH)(123-332) (see SEQ ID NO:157).

## Table 7

G GAT CCG AAC GCT ATC TTC CTG TCT TTC CAG CAC CTG CTG CGT GGC

Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly
123

AAA GTT CGT TTC CTG ATG CTG GTT GGC GGT TCT ACC CTG TGC GTT CGT Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg 140

CGG GCG CCA ACC ACT GCT GTT CCG TCT CGT ACC TCT CTG GTT CTG
Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu
160

ACC CTG AAC GAG CTC
Thr Leu Asn Glu Leu
170 174

The pBL(XH)h6T(1-163) prepared in Example 42 was digested with BamHI and HindIII, and the pBL(BH)(123-332) prepared above was similarly digested to produce a fragment of about 640 bp after which the two fragments were ligated together to obtain a clone pBL(XH)h6T(1-334). Further, the PCFM536/hMKT(1-163) prepared in Example 52 was digested with Xbal and Sfil to afford an about 270-bp fragment which was subsequently ligated with a pBL(XH)h6T(1-334) digested with the same restriction enzymes to produce a clone pBL(XH)hMKT(1-334). After the pBL(XH)hMKT(1-334) was digested with Xbal and Hindlll, an about 1040-bp fragment which codes for the mutation type human TPO amino acids 1-332 was recovered and purified, and then cloned into pCFM536 (EP-A-136490) predigested with Xbal and HindIII (E. coli JM109 previously transformed with pMW1 (ATCC No. 39933) was employed as a host.). The resultant clone was named pCFM536/hMKT(1-332), and the E. coli strain carrying this expression vector was employed as a transformant for the expression of the mutation type protein hMKT(1-332) protein. The expression plasmid pCFM536/hMKT(1-332) contains a DNA sequence shown in SEQ ID NO:14.

The above transformant was cultured in 60 ml of LB medium containing 50  $\mu$ g/ml of ampicillin and 12.5  $\mu$ g/ml of tetracycline overnight at 30°c, and the culture (25 ml) was then added to 1000 ml of LB medium containing 50  $\mu$ g/ml of ampicillin followed by shaking culture at 36°c until the OD<sub>600</sub> reaches 1.0 to 1.2. Then, after about 330 ml of LB medium at 65°c was added to the culture so that the final temperature of the culture became 42°c, the shaking culture was continued for further 3 hours at 42°c in order to induce expression of the variant human TPO, hMKT(1-332) protein, also referred to as [Met<sup>-2</sup>, Lys<sup>-1</sup>]TPO(1-332).

The obtained culture was directly subjected to SDS-PAGE analysis. In the SDS-PAGE analysis, Multigel 15/25 (Dai-ichi Chemical Co.) was employed. After electrophoresis and Coomassie Blue staining, the protein specific for the induction of expression was detected at a molecular weight of about 35 KD on the gel with regard to the transformant that the expression of the hMKT(1-332) protein was induced. In addition, following SDS-PAGE and electroblotting (using nitrocellulose membrane), the above expressed protein was reacted with the anti-HT1 peptide antibody prepared in Example 45. After staining, a band was detected at a molecular weight of approximately 35 kD thus indicating that the hMKT(1-332) was expressed.

<Example 67>
<u>Preparation of human TPO substitution derivatives.</u>
<u>their expression in COS7 cells, and identification of their activity</u>

According to the following procedures, whether several derivatives that the amino acids of human TPO were partially substituted with an other amino acid(s) have TPO activity was studied.

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The vector pSMT201 for expression in animal cells, for use in this Example, was constructed as follows.

First, the expression plasmid pXM-mEPORn which contains a cDNA for murine erythropoietin receptor (obtained from Dr. D'Andrea in Dana Farbor Cancer Institute; Cell: 57, 277-285 (1989)) was treated with restriction enzymes KpnI and EcoRI, and then electrophoresed on agarose gel to recover a pXM vector fragment wherein the cDNA for murine erythropoietin receptor was removed. Next, two oligonucleotides for introducing various cloning restriction sites into the above expression vector were synthesized using a DNA synthesizer (ABI). The oligonucleotides synthesized have the following basic sequences:

# primer-1:

5-CCTCGAGGAATTCCTGCAGCCCGGGACTAGTATCGGCTACCCCTACGACGTCCCCGACTACG CCGGCGTCCATCACCATCACCATCACTGAGCGGCCGCC-3' (SEQ ID NO:158)

primer-2:

5-AATTICGOCGOCGCTCAGTGATGGTGATGGTGATGGCGCCGCGGTAGTCGGGGACGTCGTAG GCGTAGCCGATACTAGTCCCGGGCTGCAGGAATTCCTCGAGGGTAC-3' (SEQ ID NO:159)

The two oligonucleotides were mixed and annealed to form a double stranded oligonucleotide which was thereafter ligated with the pXM vector recovered above in the presence of T4 DNA ligase (Takara-Shuzo) to give an expression vector pDMT201. To remove the murine DHFR cDNA contained in pDMT201 from the pDMT201, the vectors pDMT201 and pEF18S were separately digested with Notl and Hpal followed by agarose gel electrophoresis to recover a larger fragment from the pDMT201 vector DNA and a smaller fragment from the pEF18S vector DNA. Then, these fragments were ligated together using T4 DNA ligase (Takara-Shuzo) to produce an expression vector pSMT201. The pSMT201, as shown in Fig. 21, has an origin of replication of SV40, an

enhancer sequence, adenovirus major late promoter sequence, an adenovirus tripatite leader sequence, a splice signal sequence, an SV 40 early polyadenylation site sequence, an adenovirus VA RNA gene sequence, an origin of replication of pUC18 and a  $\beta$ -lactamase gene (Amp^r), together with the following restriction sites for ligation of a desired gene: Bglll, Pstl, Kpnl, Xhol, EcoRl, Smal, Spel and Notl sites. The pHTP illustrated in Example 30 carrying the full length human TPO cDNA was digested with EcoRl and Spel to give a full length human TPO cDNA fragment which was subsequently subcloned into a vector pSMT201 predigested similarly to produce a plasmid pSMT201-hTPO.

Using the thus obtained plasmid pSMT201-hTPO as a template, the plasmid  $\beta$ GL-TPO/pBlue which was employed as a template for the preparation of derivatives of interest was firstly constructed as follows.

In the βGL-TPO/pBlue the addition of the rabbit β-globin leader sequence to the 5'-non-translational site of human TPO was attempted by the Annweiler's method (Annweiler et al., Nucleic Acids Research, 19: 3750, 1991). For this purpose, the following two DNA strands which were chemically synthesized:

# 5-AATTCCAAGATCTCACACTTGCTTTTGACACAAC

TGTGTTTACTTGCAATCCCCCAAAACAGACCCC-3' (SEQ ID NO:160); and 5-GGGTCTGTCTGTTTTGGGGGATTGCAAGTAAACA

CAGTTGTGCAAAAGCAAGTGTGAGATCTTGG-3' (SEQID NO:161), were ligated with a fragment which had been obtained by digesting the vector Bluescript II SK+ (Toyo-boseki) with EcoRI and Smal to produce a vector  $\beta$ GL/pBlue in which the leader sequence was inserted.

PCR was conducted using the following primer sequences:

Sma-ATG: 5'-GGCCCGGGATGGAGCTGACTGAATTGCTC-3'

(SEQ ID NO:162)

(i.e., 102-122 sequence of SEQ ID NO: 7 to which Smal sequence has been linked); and

end: 5'-TCAAGCTTACTAGTCCCTTCCTGAGACAGATTCTG-3'
(SEQ ID NO:163)

(i.e., antisense of the 1140-1160 sequence of SEQ ID NO:7, to which Spel and HindIII sequences had been linked).

The PCR was carried out using 1 ng of the plasmid pSMT201-hTPO DNA as a template together with 10  $\mu$ M the synthetic primers. Using TaKaRa PCR Amplification Kit (Takara-Shuzo) and Programmable Thermal Controller (MJ Research), the PCR was conducted in a volume of 100  $\mu$ l under the following conditions: 3 cycles of 94 °c 1 min, 55 °c 2 min and 72 °c 2 min/cycle; then 20 cycles of 94 °c 45 sec, 55 °c 1 min and 72 °c 1 min/cycle. The PCR product was extracted with chloroform and then ethanol-precipitated twice followed by suspension in 100  $\mu$ l of TE buffer.

Subsequently, the PCR product was subjected to the restriction digestion with Smal and Hindlll, extracted with phenol/chloroform, and precipitated with ethanol. resultant precipitate was dissolved in 10 µl of TE buffer, after which it was subcloned into the vector \( \beta GL/pBlue \) which was previously digested with the same restriction enzymes (Competent High E. coli JM109 (Toyo-boseki) was used as a host). Of the transformant cells obtained, twenty clones were selected from which plasmid DNA's were prepared essentially as described in Sambook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press (1989). The purified plasmid DNA was identified through sequencing using Tag Dye Deoxy M Terminater Cycle Sequencing Kit (Applied Biosystems, Inc.) and 373A DNA sequencer (Applied Biosystems, Inc.). As a result, it was confirmed that the plasmid encoding the βGL-TPO/pBlue had the expected TPO cDNA sequence without any substitution over the full length thereof. Then, after the BGL-TPO/pBlue

was digested with BgIII and SpeI, the digest was extracted with phenol/chloroform and then ethanol-precipitated. The precipitate obtained was dissolved in 10  $\mu$ I of TE buffer, and it was subcloned into the vector pSMT201 which has been digested with the same enzymes (Compitent-High E. coli JM109 (Toyo-boseki) was used as a host.). In accordance with the method described in the *Molecular Cloning* (Sambook et al., supra), the plasmid DNA was prepared from the transformant cells. The resulting expression vector pSMT/ $\beta$ GL-TPO had the structure that the  $\beta$ -globin leader sequence and the human TPO cDNA were linked at the BgIII/SpeI restriction site downstream of the sprice signal sequence of the pSMT201 vector as shown in Fig. 21. This pSMT/ $\beta$ GL-TPO vector was employed for its transfection into COS cells.

For the preparation of human TPO substitution derivatives, PCR was applied in which the Ito's method was employed (Ito et al., Gene, 102:67-70, 1991). In particular, two derivatives of human TPO that the Arg-25 and His-33 of the human TPO are substituted by Asn and Thr respectively were illustratively prepared. Respectively, these derivatives can be referred to as [Asn<sup>25</sup>]TPO and [Thr<sup>33</sup>]TPO. In PCR the following primers were employed:

T7: 5'-TAATACGACTCACTATAGGGCG-3' (SEQ ID NO:164) (corresponding to T7 promoter region of Bluescript II SK+); ΔBgIII: 5'-AATTCCAAGATCACACACTTGC-3' (SEQ ID NO:165) (for substitution of BgIII recognition sequence); end: 5'-TCAAGCTTACTAGTCCCTTCCTGAGACAGATTCTG-3'

(SEQ ID NO:166)

(antisense of the 1140-1160 of SEQ ID NO:7, to which Spel and HindIII sequences have been linked);

N3: 5'-TGGGCACTGGCTCAGGTTGCTGTGAAGGACATGGG-3'

(SEQ ID NO:167)

(Arg 25 -> Asn of SEQ ID NO:7); and

O9: 5'-TGTAGGCAAAGGGGTAACCTCTGGGCA-3' (SEQ ID NO:168) (His-33 -> Thr of SEQ ID NO:7).

The first step PCR was carried out using 1 ng of the plasmid  $\beta GL\text{-TPO/pBlue}$  DNA as a template together with 10  $\mu g$  each of the synthetic primers. The combinations of the primers were: [1]  $\Delta$ Bglll and "end" primers; [2] T7 and N3; and [3] T7 and O9. Using TaKaRa PCR Amplification Kit (Takara-Shuzo) and Programmable Thermal Controller (MJ Research), the PCR was conducted in a volume of 100 µl under the following conditions: 3 cycles of 94°c 1 min, 55°c 2 min and 72°c 2 min/cycle; then 17 cycles of 94 c 45 sec, 55 c 1 min and 72 c Each of the PCR products was extracted with 1 min/cycle. chloroform and precipitated twice with ethanol, and the precipitate was dissolved in 100 µl of TE buffer. The resulting PCR products (1 µl each) were subsequently subjected to a second PCR. The combinations of the templates were: PCR products of [1]/[2] and PCR products of [1]/[3]. With regard to the primers, the combination of T7 and end was employed in two cases. After incubation at 94°c for 10 min and addition of TaKaRa Tag, the PCR was carried out under the following conditions: 3 cycles of 94°c 1 min, 55°c 2 min and 72°c 2 min/cycle; then 9 cycles of 94°c 45 sec, 55 °c 1 min and 72°c 1 min/cycle. To each of the resulting PCR products were added 1 μl of proteinase K (5 mg/ml), 2 μl of 0.5 M EDTA and 2 µl of 20% SDS, incubated at 37°c for 30 min to inactivate Taq, extracted with phenol/chloroform, and Thereafter, the precipitate dissolved in ethanol-precipitated. 20 µl of sterile water was digested with Bglll and Spel, followed by phenol/chloroform extraction and ethanolprecipitation. The precipitate thus obtained was dissolved in 10  $\mu l$  of TE buffer, and subsequently subcloned into the expression vector pSMT201 which has been previously digested with the same restriction enzymes and treated with calf intestine alkaline phosphatase (Boehringer-Mannheim) (Competent-High <u>E. coli</u>: JM109 was employed as a host.). Of the transformant cells obtained, in each case two clones were selected from which the plasmid DNAs were prepared essentially as described in the *Molecular Cloning* (Sambook et al., <u>supra</u>). The purified plasmid DNAs were sequenced using 373A DNA sequencer and Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequencing Kit (both, Applied Biosystems, Inc.).

The plasmid prepared by PCR with the use of the primers [1] and [3] contained a cDNA encoding a TPO substitution derivative (O9/TPO). The amino acid substitution of His33(CAC) by Thr(ACC) and the extension of the original C-terminus (Gly 332) by the addition of the amino acid sequence, TSIGYPYDVPDYAGVHHHHHHH (SEQ ID NO:169), were confirmed. This derivative was referred to as [Thr<sup>33</sup>, Thr<sup>333</sup>, Ser<sup>334</sup>, Hie<sup>335</sup>, Gly<sup>336</sup>, Tyr<sup>337</sup>, Pro<sup>338</sup>, Tyr<sup>339</sup>, Asp<sup>340</sup>, Val<sup>341</sup>, Pro<sup>342</sup>, Asp<sup>343</sup>, Tyr<sup>344</sup>, Ala<sup>345</sup>, Gly<sup>346</sup>, Val<sup>347</sup>, His<sup>348</sup>, His<sup>350</sup>, His<sup>351</sup>, His<sup>352</sup>, His<sup>353</sup>]TPO.

On the other hand, the plasmid prepared by PCR with the use of the primers [1] and [2] contained a cDNA encoding a TPO substitution derivative (N3/TPO). The amino acid substitutions of Arg25(AGA) by Asn(AAC) and Glu231(GAA) by Lys (AAA) and the extension of the original C-terminus(Gly332) by the addition of the amino acid sequence, TSIGYPYDVPDYAGVHHHHHH, were confirmed. This derivative was referred to as [Asn25, Lys231, Thr333, Ser334, Ile335, Gly336, Tyr337, Pro338, Tyr339, Asp340, Val341, Pro342, Asp343, Tyr344, Ala345, Gly346, Val347, His348, His349, His350, His351, His352, His353]TPO.

The transfection into COS7 cells of each clone obtained was carried out by the DEAE-dextran method including chloroquine treatment as described in Example 35. Briefly, 40 µg each of the plasmid DNA's were used in transfection, and, after 5 days, the culture supernatant was recovered which

were subsequently concentrated to a volume of 1/20 using Centricon-30 (Amicon) in order to evaluate their TPO activity by the M-O7e assay. As a result, in the culture supernatants of COS7 cells into which plasmids containing cDNAs encoding the human TPO substitution derivatives N3/TPO and O9/TPO were transfected respectively, the TPO activity was detected in a dose-dependent manner (see Fig. 22).

## <Example 68>

Preparation of insertion or deletion derivatives of human TPO, expression thereof in COS7 cells and identification of TPO activity

This Example makes evident whether insertion or deletion derivatives of hTPO163 have a TPO activity.

Derivatives prepared were a His33-deletion derivative ([ΔHis<sup>33</sup>]TPO(1-163), dH33); a Gly116-deletion derivative ([ΔGly<sup>116</sup>]TPO(1-163), dG116); an Arg117-deletion derivative ([ΔArg<sup>117</sup>]TPO(1-163), dR116); a Thr-insertion derivative ([His<sup>33</sup>, Thr<sup>33'</sup>, Pro<sup>34</sup>]TPO(1-163), T33'), an Alainsertion derivative ([His<sup>33</sup>, Ala<sup>33'</sup>, Pro<sup>34</sup>]TPO(1-163), A33') and a Gly-insertion derivative ([His<sup>33</sup>, Gly<sup>33'</sup>, Pro<sup>34</sup>, Ser<sup>38</sup>]TPO(1-163), G33') in which Thr, Ala and Gly have been independently inserted between His33 and Pro<sup>34</sup>; and an Asninsertion derivative ([Gly<sup>116</sup>, Asn<sup>116'</sup>, Arg<sup>117</sup>]TPO(1-163), N116'), an Ala-insertion derivative ([Gly<sup>116</sup>, Ala<sup>116'</sup>, Arg<sup>117</sup>]TPO(1-163), A116') and a Gly-insertion derivative ([Gly<sup>116</sup>, Gly<sup>116'</sup>, Arg<sup>117</sup>]TPO(1-163), G116') in which Asn, Ala and Gly have been independently inserted between Gly116 and Arg117, all the sequences being based on SEQ ID NO:13.

Among the derivatives, T33' and N116' are intended to introduce mucin type and Asn-binding type sugar chains, respectively.

The above mentioned derivatives were prepared using PCR by the method described by Ito et al (Gene, 102: 67-70, 1991). Primer sequences used in PCR are as follows:

dH33: 5'-TGTAGGCAAAGGAACCTCTGGGCA-3' (SEQ ID NO:172) (for the preparation of His33-deletion derivative based on the sequence shown in SEQ ID NO:7);

T33': 5'-TGTAGGCAAAGGAGTGTGAACCTCTGG-3' (SEQ ID NO:173) (for the preparation of Thr-insertion derivative with Thr inserted between His33 and Pro34 in the sequence shown in SEQ ID NO:7);

A33': 5'-TGTAGGCAAAGGAGCGTGAACCTCTGG-3' (SEQ ID NO:174) (for the preparation of Ala-insertion derivative with Ala inserted between His33 and Pro34 in the sequence shown in SEQ ID NO:7);

G33': 5'-TGTAGGCAAAGGTCCGTGAACCTCTGG-3' (SEQ ID NO:175) (for the preparation of Gly-insertion derivative with Gly inserted between His33 and Pro34 in the sequence shown in SEQ ID NO:7);

dG116: 5'-AGCTGTGGTCCTCTGTGGAGGAAG-3' (SEQ ID NO:176) (for the preparation of Gly116-deletion derivative based on the sequence shown in SEQ ID NO:7);

dR117: 5'-GTGAGCTGTGGTGCCCTGTGGAGG-3' (SEQ ID NO:177) (for the preparation of Arg117-deletion derivative based on the sequence shown in SEQ ID NO:7);

N116': 5'-AGCTGTGGTCCTGTTGCCCTGTGGAGG-3' (SEQ ID NO:178) (for the preparation of Asn-insertion derivative with Asn inserted between Gly116 and Arg117 in the sequence shown in SEQ ID NO:7);

A116': 5'-AGCTGTGGTCCTAGCGCCCTGTGGAGG-3' (SEQ ID NO:179) (for the preparation of Ala-insertion derivative with Ala inserted between Gly116 and Arg 117 in the sequence shown in SEQ ID NO:7);

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G116': 5'-AGCTGTGGTCCTTCCGCCCTGTGGAGG-3' (SEQ ID NO:180) (for the preparation of Gly-insertion derivative with Gly inserted between Gly116 and Arg117 in the sequence shown in SEQ ID NO:7);

dri: 5'-CGGGCTGCAGGATATCCAAGATCTCA-3' (SEQ ID NO:181) (for the substitution of a restriction enzyme EcoRI-recognition sequence);

5'-TAATACGACTCACTATAGGGCG-3' (SEQ ID NO:182) T7: (corresponding to T7 promoter region of BluescriptIISK+); and endNotl: 5'-GGGCGGCCGCTCAGCTGGGGACAGCTGTGGTGGG-3' (SEQ ID NO:183) (antisense of the 633-653 nucleotide sequence of SEQ ID NO:7, to which a termination codon and a Notl recognition sequence have been added).

A first step PCR was carried out using, as a template, 1 ng of the plasmid βGL-TPO/pBlue DNA prepared in Example 67 and using 10  $\mu M$  synthetic primers. combination of the primers was: [1] dRI and endNotl or [2] T7 and mutated primers (dH33, A33', G33', T33', dG116, dR117, A116', G116' and N116'). In PCR, TaKaRa PCR Amplification Kit (Takara-Shuzo) and Programmable Thermal Controller (MJ Research) were employed, and the PCR was carried out in a final volume of 100  $\mu$ l. In the first PCR reaction of [1], one cycle was 94 c 1 min, 45 c 2 min and 72 c 2 min and 3 cycles were conducted, followed by 17 cycles in which one cycle was 94 ° c 45 sec, 45 ° c 1 min and 72 ° c 1 min. On the other hand, the first PCR reaction of [2] was carried out under the conditions of 94 c 1 min, 55 c 2 min and 72 c 2 min for 3 cycles followed by 94 c 45 sec, 55 c 1 min and 72 c 1 min for Each of the PCR products was extracted with 17 cycles. chloroform before twice ethanol precipitation, and then the resultant precipitate was dissolved in 100  $\mu$ l of TE buffer.

Next, a second step of the PCR was conducted using 1 ul of each of the PCR products of [1] and [2]. The primers used were a combination of T7 and endNotl. After incubation at

94 °C for 10 min, to each PCR reaction mixture was added TaKaRa Taq. The second PCR was carried out under the conditions of 94 c 1 min, 55 c 2 min and 72 c 2 min for 3 cycles followed by 94 c 45 sec, 55 c 1 min and 72 c 1 min for 9 cycles. To each PCR product thus obtained were added 1  $\mu l$ of 5 mg/ml proteinase K, 2  $\mu l$  of 0.5 M EDTA and 2  $\mu l$  of 20% SDS, and the mixture was kept at a temperature of 37°c for 30 min to inactivate Taq. The PCR product was extracted with phenol/chloroform followed by ethanol-precipitation, and the resultant precipitate was then dissolved in 20 μl of sterile water. After digested with EcoRI and NotI, the solution was extracted with phenol/chloroform and then precipitated with ethanol. The obtained precipitate was dissolved in 10 µl of TE buffer, and subcloned into an expression vector pEF18S that has been previously digested with the same restriction enzymes and treated with alkaline phosphatase (from calf intestine, Boehringer-Mannheim). A host cell employed was Competent-High E. coli JM109 (Toyo-Boseki). transformant thus obtained, a plasmid DNA was prepared essentially by the method described in Molecular Cloning (Sambook et al., Cold Spring Harbor Laboratory Press (1989)). Thereafter, the nucleotide sequence of the purified plasmid DNA was confirmed using Taq Dye Deoxy<sup>TM</sup> Terminater Cycle Sequencing Kit (Applied Biosystems) and using an Applied Biosystems 373A DNA sequencer. As a result, it was confirmed that all the plasmids encoding dH33, A33', T33', dG116, dR117, A116', G116' and N116' had the expected TPO cDNA sequence without any substitution of a nucleotide sequence at sites other than the intended sites. Moreover, in G33' a base substitution [Pro38 (CCT)->Ser (TCT)] other than that at the intended site was observed.

The transfection of each clone obtained into COS7 cells was conducted in accordance with the method in Example 11. Briefly, the transfection was carried out using 10  $\mu g$  of

each plasmid DNA by the DEAE-dextran method including chloroquine treatment, and after 4 to 5 days, the culture supernatant was recovered which was then evaluated by M-07e assay system. As a result, in each supernatant of the COS7 cell cultures transfected with plasmids encoding an amino acid insertion or deletion derivative, the TPO activity was detected in a dose-dependent manner (see Fig. 23). On the contrary, no TPO activity was found in the supernatant of the COS7 cell culture that the expression plasmid pEF18S not containing a cDNA encoding a TPO derivative has been transfected into and expressed.

# <Example 69>

# Preparation and purification of anti-human TPO peptide antibodies

(1) Six regions (shown in Table 8) which are considered relatively suitable as antigens were selected from the amino acid sequence of human TPO of SEQ ID NO: 191, from which a four-stranded peptide of a multiple antigen peptide (MAP) type was synthesized by Tam's method (*Proc. Natl. Acad. Sci., USA, 85*, 5409-5413, 1988). Two rabbits were immunized eight times each with 100 μg of this peptide.

Apart from the above, single-stranded peptides were produced by bonding cysteine residue to the C-terminal of each of the peptide regions shown by SEQ ID NOS: 119-124 (these are referred to as peptide region HT 1 to 6 regions, respectively, which are partial peptides corresponding to 8 to 28, 47 to 62, 108 to 126, 172 to 190, 262 to 284, and 306 to 332, respectively, of the sequence of SEQ ID NO: 191). Using these as test antigens, the antibody values thereto in the sera obtained from the thus-immunized rabbits were measured by enzyme-immunoassay, from which the increase in the antibody value was confirmed in all the sera tested. Thus, these sera were referred to as anti-sera.

In addition, the above-mentioned single-stranded synthetic peptide having the cysteine residue bonded to its C-terminal was used also as the antigen for the affinity purification of the anti-sera in the following (2).

(2) Two rabbits each were immunized with one of the above-mentioned antigen peptides, and the antibody derived from each of all the thus-immunized rabbits was prepared separately. Concretely, for anti-HT1 peptide antibodies, obtained were anti-HT1-1 peptide antibody and anti-HT1-2 peptide antibody derived from the two immunized rabbits.

As one example, the purification of anti-HT1-1 peptide antibody is illustrated hereunder.

First, 30 mg of the single-stranded peptide of HT1 having cysteine residue bonded thereto were coupled onto 12 ml of Sulfo Link Coupling Gel (produced by Pierce Co., as catalog No. 44895). Precisely, a peptide solution containing the antigen was coupled onto the gel that had been equilibrated with a coupling buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5) of 6 times by volume the gel, over a period of 15 minutes. Next, after having been left to statically stand as it was for 30 minutes, the gel was washed with the coupling buffer of 3 times by volume the gel. Next, the coupling buffer containing 0.05 M L-cysteine-HCl was added to the gel at a rate of 1 ml/ml-gel, by which the non-treated groups were blocked over a period of 15 minutes. After having been left to statically stand as it was for 30 minutes, the gel was washed with the coupling buffer of 8 times by volume the gel. mentioned coupling operation was conducted at room In this way, the antigen region-containing peptide was coupled onto the gel by covalent bonding, at a coupling efficiency of 28.3 %, to prepare an antigen peptide gel having 0.8 mg of the peptide coupled onto 1 ml of the gel, in the antigen column. After collecting all blood from each

immunized rabbit, 78.4 ml of anti-serum containing anti-HT1-1 peptide antibody were obtained. 76.7 ml of the anti-serum (having a protein content of 3620 mg) were added to the antigen column that had previously been equilibrated with 50 mM phosphate buffer (pH 8) containing 150 mM of NaCl and 0.05 % of sodium azide, then the column was washed with the same buffer. Thus, 105.9 ml of a passing fraction (having a protein content of 3680 mg) were obtained.' Next, the fraction adsorbed was eluted with 0.1 M citric acid buffer (pH 3.0), which was then immediately neutralized by adding thereto 21.1 ml of 0.1 M carbonate buffer (pH 9.9), concentrated by ultrafiltration (using YM30 membrane produced by Amicon Co.) and purified in 50 mM phosphate buffer (pH 8) containing 150 mM of NaCl and 0.05 % sodium azide. Thus, 11.2 ml of a purified anti-HT1-1 peptide antibody (having a protein content of 77.7 mg) were obtained in the buffer. In the same manner as above, anti-HT1-2 peptide antibody (60.0 mg), anti-HT2-1 peptide antibody (18.8 mg), anti-HT2-2 peptide antibody (8.2 mg), etc. were obtained.

Anti-HT3 to anti-HT6 peptide antibodies are obtained in the same manner as above.

# <Example 70>

#### Detection of TPO Western Analysis

(1) To evaluate the anti-sera obtained in Example 69, these were tested in the manner mentioned below.

A standard sample of recombinant human TPO that had been partially purified from the supernatant of the culture of CHO cells, into which a gene coding for the amino acids -21 to 332 of SEQ ID NO:6 had been introduced and expressed, was subjected to SDS-polyacrylamide electrophoresis (SDS-PAGE) according to an ordinary method and then electrically blotted on a PVDF or nitrocellulose membrane. After the blotting, the membrane was washed with 20 mM Tris-HCl and 0.5 M NaCl (pH

7.5) (TBS) for 5 minutes, then washed two times each with 0.1 % Tween 20-containing TBS (TTBS) for 5 minutes, and treated with a blocking agent (Block Ace; produced by Dai-Nippon Pharmaceutical Co., as catalog No. UK-B25) for 60 minutes. The anti-sera each separately containing one of anti-HT1-1 peptide antibody, anti-HT2-1 peptide antibody, anti-HT3-2 peptide antibody, anti-HT4-1 peptide antibody, anti-HT5-2 peptide antibody and anti-HT6-1 peptide antibody each were diluted with TTBS solution containing 0.05 % BSA and 10 % Block Ace to 1/1000 dilutions. The thus-diluted antibodies were employed for Western assay as the primary antibodies. Precisely, the recombinant human TPO sample processed as above was treated with TTBS solution containing the anti-TPO peptide antibody, 0.05 % BSA and 10 % Block Ace for 60 minutes and then washed with TTBS for 5 minutes. Next, this was treated with TTBS solution containing anti-rabbit(ab') goat biotinated antibody (produced by Caltag Co., as catalog No. L43015), as the secondary antibody, along with 0.05 % BSA and 10 % Block Ace, for 60 minutes, and then washed two times each with TTBS for 5 minutes. Next, this was treated with a 1/5000 dilution of alkaline phosphatase-labeled avidin (produced by Leinco Technologies Co., as catalog No. A108) diluted with 10 % Block Ace-containing TTBS solution, for 30 minutes, and thereafter washed two times each with TTBS for 5 minutes and then with TBS for 5 minutes. Then, this was developed, using an alkaline phosphatase substrate (produced by Bio-Rad Co., as catalog No. 170-6432). mentioned Western assay was conducted at room temperature, which verified that the human TPO was recognized and detected by the anti-sera.

(2) 3 mg of each of anti-HT1-1 peptide antibody, anti-HT1-2 peptide antibody, anti-HT2-1 peptide antibody and anti-HT2-2 peptide antibody that had been purified by affinity chromatography in Example 69 were weighed and biotinated by

coupling them onto an activated biotin (NHS-LC-Biotin II, produced by Pierce Co., as catalog No. 21336). The same standard sample of recombinant human TPO as that used in the foregoing (1) was subjected to SDS-PAGE, then electrically blotted on a PVDF or nitrocellulose membrane, and subjected to ordinary Western assay using each of these biotinated antibodies as the primary antibodies. Immediately after the blotting, the membrane was washed with TBS for 5 minutes and then washed two times each with TTBS for 5 minutes, and thereafter this was treated with a blocking agent (Block Ace) for 60 minutes. Next, this was treated with TTBS solution containing 1 µg/ml of the biotinated anti-TPO peptide antibody, 0.05 % BSA and 10 % Block Ace for 60 minutes and then washed two times each with TTBS for 5 minutes. Next, this was treated with a 1/5000 dilution of alkaline phosphatase-labeled avidin (produced by Leinco Technologies Co., as catalog No. A108) diluted with 10 % Block Acecontaining TTBS solution, for 30 minutes, and thereafter washed two times each with TTBS for 5 minutes and then with TBS for 5 minutes. Then, this was developed, using an alkaline phosphatase substrate (produced by Bio-Rad Co., as catalog No. The above-mentioned Western assay was 170-6432). conducted at room temperature, which verified that the human TPO was recognized and detected by the purified antibodies.

# <Example 71> <u>Preparation of anti-TPO peptide</u> antibody columns and detection of human TPO

The anti-human TPO peptide antibodies obtained in Example 69 were verified to recognize human TPO. These antibodies were separately coupled onto a chromatography carrier to prepare anti-TPO peptide antibody columns. As one example, the preparation of anti-HT1-2 peptide antibody column is illustrated hereinafter.

(1) A solution comprising 50 mM Na phosphate and 0.15 M NaCl (pH 8.0) and containing 5 mg/ml of the antibody was prepared. 0.8 ml of this solution were combined and coupled with 1.54 ml of a swollen formyl-activated gel (Formyl-Gellulofine, produced by Chisso Co.) at 4 c for 2 hours. Next, 1.1 ml of a solution containing 10 mg/liter of a reducing agent (trimethylamine borane (TMAB), produced by Seikagaku Kogyo K.K., as catalog No. 680246) we're added thereto and coupled with them for further 4 hours. Then, only the gel part was recovered from the reaction mixture by centrifugation. 10 ml of pure water were added thereto and, only the gel part was recovered from this by centrifugation. This process was repeated four times, by which the non-reacted antibody was removed. Next, the thus-recovered gel was treated with 2.1 ml of a blocking buffer (0.2 M Na phosphate, 1 M ethanolamine, pH 7.0) and 1.1 ml of the solution of reducing agent that had been added thereto, at 4°c for 2 hours, by which the active groups in the non-reacted gel were blocked. Last, the resulting gel was washed with water, 20 mM Tris-HCl and 0.15 M NaCl (pH 8.0), using a centrifuge. This was filled in a small column tube, which was washed with 3 M sodium thiocyanate solution and 0.1 M glycine-HCI (pH 2.5) solution and then again equilibrated with 20 mM Tris-HCl and 0.15 M NaCl (pH 8.0). The thusequilibrated column was stored.

The anti-TPO peptide antibody gel in the anti-HT1-2 antibody column had a coupling efficiency of up to 94.2 % for each IgG fraction. The amount of the IgG fraction coupled onto the gel per the unit volume of the gel was 1.9 mg/ml-gel. In the same manner as above, a gel was prepared, onto which 21.8 mg, per ml of the gel, of IgG not having TPO as the antigen had been coupled. This was used as a pre-column (hereinafter referred to as a pre-antibody column) for the purpose of removing the non-specifically-bonding molecules from TPO antibody columns, as is mentioned in (2).

(2) Next, another example of preparing an anti-HT1-2 antibody column is illustrated hereinafter.

A standard sample of recombinant human TPO that had been partially purified from the supernatant of the culture of CHO cells into which a gene coding for the amino acid sequence of any of SEQ ID NOS: 119-124 had been introduced and expressed was added to the pre-antibody column (containing 1 ml of gel) prepared in (1), and 10 times by volume the gel of 20 mM Tris-HCl (pH 8.0) and 0.15 M NaCl were passed through the column, whereupon the fraction passing through the column was collected. Next, the fraction adsorbed to the pre-antibody column was eluted with 10 times by volume the gel of an acidic eluent (0.1 M glycine-HCl, pH Next, the fraction that had passed through the preantibody column was added to the anti-HT1-2 antibody column (containing 2 ml of gel) prepared in (1), and the column was washed with 10 times by volume the gel of 20 mM Tris-HCl (pH 8.0) and 0.15 M NaCl, while collecting the fraction passing through the anti-HT1-2 antibody column. Next, the fraction adsorbed to the anti-HT1-2 antibody column was eluted with 8 times by volume the gel of an acidic eluent (0.1 M glycine-HCl, pH 2.5). These fractions were analyzed by SDS-PAGE, which verified that TPO did not adsorb to the pre-antibody column but was specifically bonded to the anti-HT1-2 antibody column and that almost all TPO in the sample added bonded the latter column. From this, it was confirmed that at least from 200 to 300 µg/ml-gel of TPO was bonded to the gel in the latter column.

#### <Example 72>

# Effect of TPO for Increasing the Numbers of Platelets

Twenty normal ICR strain male mice, eight weeks of age, (blood was collected from their orbit veins already and the numbers of platelets was measured using a micro cell

counter F-800 manufactured by Toa Iyo Denshi) were randomly divided into four groups. One of the four groups (a control-iv group) was injected with 100 microliters of PBS intravenously route once daily for five consecutive days. One of the other groups (TPO-iv group) was injected with 100 microliters of a solution (prepared by substituting the concentrated solution of the TPO active fraction F2 of the SP Sepharose Fast Flow obtained in Example 56 with PSB using an NAP-25 column [Pharmacia Biotech; Catalog No. 17-0852-02] followed by diluting with PBS; containing relative activity, 211,900 by M-07e assay) intravenously once daily for five consecutive days. Another group (control-sc group) was injected with 100 microliters of PBS subcutaneously once daily for five consecutive days while the remaining group (TPO-sc group) was injected with 100 microliters once daily for five consecutive days in the same manner as in the TPO-iv group After 6, 8, 10, 13 and 15 days from the subcutaneously. administrations, blood was collected from initiation of the the orbit vein of each of the mice and the numbers of platelets was counted using a micro cell counter (F-890; manufactured by Toa Iyo Denshi). The changes in the numbers of platelets are given in Fig. 24. Thus, in the control-iv group, the platelet number increased by 44% as compared with before the administration even on the Day 6 (Day 0 being the day of administration) where the platelet numbers were highest and, in the control-sc group, only a 47% increase was noted even on Day 8 where the numbers were highest. On the other hand, in the TPO-iv group, a 177% increase was noted on Day 6 as compared with the numbers of prior to the administration and, in the TPO-sc group, an increase of 347% was noted already on Day 6 and, on Day 8, the highest increase of 493% was achieved.

From the above results, it has been confirmed that human TPO increases the number of platelets independently of

the difference in the type and also of the route of administration.

#### <Example 73>

# Effect of TPO for Increasing the Number of Platelets

Sixteen normal C3H/HeJ strain male mice, 11 weeks of age, (the blood was already collected from their orbit veins and the number of platelets were measured using a microcell counter type F-800 manufactured by Toa Iyo Denshi) were randomly divided into four groups. One of the four groups (a control group) was injected with 100 microliters of PBS subcutaneously once daily for five consecutive days. Another group (TPO-1 group) was injected subcutaneously with 100 microliters of a solution (prepared by substituting the TPO active fraction of Sepha cryl S-200HR obtained in Example 56 NAP-25 followed by diluting with PBS; with PBS using containing relative activity, 83,000 by the M-07e assay) once daily for five consecutive days. Still another group (TPO-2 group) was injected with 100 microliters of a solution (prepared by diluting the TPO active fraction used in the TPO-1 group with PBS to an extent of 2-fold; containing relative activity, 41,500 by the M-07e assay) subcutaneously once daily for five consecutive days. The last group (TPO-3 group) was injected with 100 microliters of a solution (prepared by diluting the TPO active fraction used in the TPO-2 group to an extent of 2-fold more; containing relative activity, 20,750 by the M-07e assay) subcutaneously once daily for five consecutive days.

Blood was collected from the orbit veins of the mice on Day 6, 8, 10 and 12 after injection and the numbers of platelets were counted using a microcell counter (type F-800; manufactured by Toa Iyo Denshi).

The changes in the numbers of platelets are given in Fig. 25. Thus, in the control group, there was nearly no

change in the numbers of platelets while, in the groups to which TPO was injected, there were increases in the numbers of platelets in all of the groups to the extent that the numbers were highest on Day 8 after the injection. In addition, differences in dose-response were noted among the groups. Thus, in the TPO-1 group, about a 200-% increase was noted on Day 6 already, the increase rose to about 270% on Day 8 and then, however, a decrease was noted, even on Day 12, about a 65-% increase was still noted whereupon there was a significant difference from the control group (p < 0.01; by a Dunnet multiple comparison test). In the TPO-2 group, the same increase was found and, though the increasing action was smaller than the TPO-1 group, increases of about 140% and about 160% were noted on Days 6 and 8, respectively. On Day 12, the numbers decreased to nearly the same level as the control group. The increasing action of the TPO-3 group was weaker than the TPO-2 group and, on Day 8 where the numbers were highest, about a 110-% increase was noted and, again, there was a significant difference (p < 0.01) from the control group.

From the above-mentioned results, it has been confirmed that the human TPO increases the platelet numbers independently of the strain of the mice and that its action has a dose-responding property.

# <Example 74> An Inhibiting Effect of TPO on the Thrombocytopenia Caused by Administration of Anti-Cancer Agent

Thirty ICR male mice, eight weeks of age, were injected with 200 mg/kg of 5-FU intravenously and randomly divided into two groups (each group comprising 15 mice). From the next day (Day 1), one of the groups (control group) was injected with 100 microliters of PBS subcutaneously once daily for five consecutive days while another group (TPO

group) was injected with 100 microliters of TPO active fraction used in Example 72 (containing relative activity, 211,900 by the M-07e assay) subcutaneously once daily for five consecutive days. On Days 4, 6 and 8, each five mice were randomly chosen from each of the groups, then blood was collected from the orbit veins and the numbers of platelet were counted by a microcell counter (type E-2500; manufactured by Toa Iyo Denshi). The changes in the numbers of platelets are given in Fig. 26. Thus, in the control group, the administration of 5-FU numbers of platelets after the decreased as the days passed and, on Day 6, the value was lowest (about 28% of the value prior to the administration of 5-FU) though, on Day 8, an approximately 1.3-fold increase was noted as a reaction of the decrease. Even in the case of a TPO group, the numbers of platelets after the administration of 5-FU decreased as the days passed and, on Day 6, the value was the lowest (about 52% of the value before the administration of 5-FU). However, the difference between the numbers of platelets on Days 4 and 6 was very small and, on Day 6, a significantly (p < 0.05; by a Dunnet multiple comparison test) value as compared with the control group was maintained. On Day 8, there was an increase of about 200% of the value before the 5-FU administration.

From the above-mentioned result, it has been confirmed that human TPO inhibits the decrease of the platelet number as caused by the anti-cancer agent.

#### <Example 75>

# A Therapeutic Effect of TPO for Thrombocytopenia

Nimustine hydrochloride (ACNU) (50 mg/kg) was injected intravenously to each of 36 ICR male mice, seven weeks of age, and then the mice were randomly divided into two groups (each group comprising 18 mice). As from the next day (Day 1), one of the groups (control group) was injected

with 200 microliters of PBS subcutaneously twice daily for consecutive days while another group (TPO group) was injected with 200 microliters of the TPO active fraction used in Example 73 (without diluting with PBS) to which 0.04% of Tween 80 was added (containing relative activity, 380,000 by the M-07e assay) subcutaneously twice daily for consecutive days.

After the injection, the mice in each of the group randomly divided into three groups - from one group blood was collected on Days 5 and 12; from another group on Days 8 and 14; and from the remaining group on Day 10. Blood veins and the numbers of was collected from the orbit platelet number were counted by a microcell counter (type F-800; manufactured by Toa Iyo Denshi). The changes in the numbers of platelets are given in Fig. 27. Thus, in the control group, the numbers of platelets after administration of ACNU decreased as the days passed and, on Days 8-10, the value was lowest (about 29% of the value before the ACNU administration) and the recovery was only about 49% and about 74% on Days 12 and 14, respectively. On the other hand, in the TPO group, a decrease to about 38% of the value before the ACNU administration was noted on Day 5 and then the situation changed to an increase. Thus, on Day 8, the value recovered to about 63% of the value before the ACNU administration and, on Day 10 and thereafter, the numbers of platelets were larger than that before the ACNU administration. Thus, on Days 12 and 14, increases of up to about 300% and about 400% were noted, respectively.

As noted hereinabove, on Days 8-10 of which the decrease was noted in the control group, an increase was observed in the TPO group already and, on Day 10, the platelet numbers were more than those before the administration of ACNU. Accordingly, it has been confirmed that human TPO

promotes the recovery from the thrombocytopenia caused by the anti-cancer agent.

When the results of Example 74 are taken into consideration as well, it is now expected that human TPO inhibits the decrease in the numbers of platelets and promotes the recovery from thrombocytopenia independently of the type of the anti-cancer agent.

### <Example 76>

# A Therapeutic Effect of TPO on Thrombocytopenia after BMT

Forty-eight male mice of C3H/HeN strain, seven weeks of age, were irradiated with 10 Gy of radioactive radiation on the entire body and, immediately thereafter, they were intravenously injected with 1 x 10<sup>6</sup> of bone marrow cells from the mice of the same strain. The mice were then randomly divided into two groups and, from the next day (Day 1), one of the groups (control group) was injected with the PBS while another group (TPO group) was injected with the TPO active fraction used in Example 73 (containing relative activity, 44,000 by the M-07e assay) with a dose of 100 microliters each subcutaneously once daily for twenty consecutive days.

After initiation of the injection, each six mice were randomly selected on Days 5, 10, 14 and 21, blood was collected from their orbit veins and the numbers of platelets were counted by a microcell counter (type E-2500; manufactured by Toa Iyo Denshi).

The changes in the numbers of platelets are given in Fig. 28. Thus, in all of the groups, the numbers of platelets decreased as the days passed after applying the BMT thereto and, on Day 10, the numbers were lowest (about 3% of the numbers of before the BMT application). After that, a gradual recovery was noted and, on Day 4, the numbers were about 11% and about 13% in the control group and in the TPO

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group, respectively. On Day 21, only a 37% recovery was noted in the control group while, in the TPO group, the recovery was about 65% whereupon a significant recovery promoting effect was observed. From the above-mentioned results, it has been confirmed that human TPO prepared as in Example 56 promote the recovery of the numbers of platelets after application of the BMT.

<Example 77>

# A Therapeutic Effect of TPO for

# Thrombocytopenia after Irradiation with Radioactive Ray

Thirty-six male mice of ICR strain, eight weeks of age, were irradiated with x-rays of 5 Gy on the entire body and randomly divided into two groups. From the next day (Day 1), one of the groups (control group) was injected with PBS while another group (TPO group) was injected with a TPO active fraction used in Example 73 (containing relative activity, 1,440,000 by the M-07e assay) once daily for three consecutive days subcutaneously and, from the next day, relative activity, 360,000 by the M-07e assay was injected subcutaneously once daily for seven consecutive days. After initiation of the injection, each of the group was randomly divided into three groups - a group from which blood was collected on Days 4, 11 and 21; another group on Days 7 and 13; and the remaining group on Days 9 and 15. The collection of blood was carried out from the orbit veins and the numbers of platelets were counted by a microcell counter (type F-800; manufactured by Toa Iyo Denshi). The changes in the numbers of platelets are given in Fig. 29. Thus, in the control group, the numbers of platelets decreased as the days passed after irradiation with x-rays and, on Day 9, the numbers were smallest (about 25% of the number before the irradiation with X-rays). On days 11 and 13, the numbers recovered to about 38% and about 67%, respectively and, on Day 15, they recovered

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to the values before the irradiation with x-rays. In the TPO group, the numbers decreased to about 24% of those before the irradiation with X-rays on Day 7. After that, an increase was noted and, on Day 9, the numbers recovered to about 82%. On Day 11 and thereafter, the platelet numbers were more than those before irradiation with X-rays and, until Day 21, this tendency was maintained. As mentioned above, on Day 9 on which the numbers tended to decrease in the control group, the numbers of platelets in the TPO group already changed to an increase and, on Day 11, the numbers of platelets were more than those before the irradiation with X-rays while remaining high even thereafter. On the other hand, in the control group, fifteen days were needed for the numbers of platelets to recover from before the irradiation with X-rays. results, it has been confirmed that human TPO produced in Example 56 shortens the time necessary to recover from the decrease in the numbers of platelets after irradiation with Xrays and that it exhibits an therapeutic effect for thrombocytopenia after the irradiation with X-rays.

#### <Example 78>

#### Effect of hTPO163 for Increasing the Numbers of Platelets

Fifteen healthy male mice of C3H/HeN strain from whom blood was already collected from their orbit veins and subjected to the number of platelets was measured by a microcell counter (type F-800; manufactured by Toa Iyo Denshi) were randomly divided into four groups - a control group and groups A, B and C. The first group (control group) was injected subcutaneously with PBS containing 0.1% of serum from mouse once daily for five consecutive days. Groups A, B and C were injected with the TPO active fraction of SP Sepharose Fast Flow obtained in Example 65 followed by diluting with PBS containing 0.1% of mouse serum at the doses of about 40,000,000, about 8,000,000 and about

1,600,000/kg body weight/day, respectively, in terms of the relative activity of hTPO163 by the M-07e assay for five consecutive days subcutaneously.

On Days 6, 8, 10 and 12 after the injection, blood was collected from the orbit vein of each mouse and the numbers of platelets were counted by a microcell counter (type F-800; manufactured by Toa Iyo Denshi). The changes in the numbers of platelets are given in Fig. 30.

Thus, in the control group, there was nearly no change in the numbers of platelets while, in the TPO-given groups, increases in the numbers of platelets were noted in all cases giving the highest values on Day 8 after the injection. Among each of the TPO-given groups, a dose-response relationship was noted. Thus, in group A, increases of about 88% and about 100% were noted on Days 6 and 8, respectively and, even on Day 10, an increase of about 97% was maintained whereupon all data showed a significant difference from the control group (p < 0.01 by a Dunnet Multiple Comparison Test). Similar increases were noted in group B as well and, though the degree of increase was lower than that of group A, the increases of about 65% and about 84% were noted on Days 6 and 8, respectively showing a significant difference from the control group (p < 0.01 or 0.05 by a Dunnet Comparison Test). The increasing action in group C was lower than that in group B. Still it showed about 31% increase on Day 8 when the highest numbers were noted. From the abovementioned results, it has been confirmed that the hTPO163 produced in Example 65 increases the numbers of platelets and that its action shows a dose-response relationship.

#### <Example 79>

# A Therapeutic Effect of hTPO163 for Thrombocytopenia

One hundred male mice of C3H/HeN strain, eight weeks of age, were injected intravenously with 50 mg/kg of

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nimustine hydrochloride (ACNU) by and then randomly divided into four groups - a control group and groups A, B and C - each comprising 25 mice. As from the next day (Day 1), the control group was injected subcutaneously with 5 ml/kg of PBS once daily for consecutive days while groups A, B and C were injected subcutaneously with the TPO active fraction of the SP Sepharose Fast Flow obtained in Example 65 diluted with PBS at the doses of about 16,000,000, about 48,000,000 and about 160,000,000/kg body weight/day, respectively, in terms of the relative activity of the hTPO163 by the M-07e assay once daily for consecutive days.

After initiation of the injection, mice in each of groups were randomly divided into five groups and the collected on Days 5, 8, 10, 12 and 14. collection of blood was carried out from their orbit veins and the numbers of platelets were counted by a microcell counter (type E-2500; manufactured by Toa Iyo Denshi). The changes in the numbers of platelets are given in Fig. 31. Thus, in the control group, the numbers of platelets decreased as the days passed after the administration of ACNU giving the values (about 15-16% of that before the ACNU administration) on Days 8-10 and the extent of the recovery was only as little as about 28% and about 51% on Days 12 and 14, respectively. On the other hand, in group A, the numbers decreased to about 25% of those before the ACNU administration on Day 8. After that, however, they changed to increase and recovery to an extent of about 34% and about 89% were noted on Days 10 and 12, respectively. On Day 14, the number was larger than that of before the ACNU administration.

As mentioned above, the smallest numbers of platelets were noted on Day 8 in all of the groups though, in the groups to which human TPO was given, the decrease was slower than the control group giving rise to the smallest numbers of platelets. In the control group, there was nearly

no recovery of the numbers of platelet even on Day 10 while, in the groups given the human TPO, recovery in the numbers of platelets were noted in all of the groups, though their degrees varied. In a group to which 50 micrograms/kg was administered, recovery was noted on Day 12 already giving higher numbers than of those before the ACNU administration. When it is compared with the fact that, in the control group, the recovery was as small as about 51% as compared with the state before the ACNU administration even on Day 14, it is now clear that the hTPO163 produced as in Example 65 promotes the recovery from the decrease in the numbers of platelets. As such, the hTPO163 has been confirmed to have a therapeutic effect on thrombocytopenia caused by anti-cancer agents.

Examples of the pharmaceutical preparations are hereinunder.

# <Example 80>

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The human TPO fraction obtained in Example 56 was concentrated, subjected to aseptic treatment and frozen at -20°c to give a frozen product to be used as an injectable preparation.

#### <Example 81>

The human TPO fraction obtained in Example 56 was concentrated, 5 ml of it was filled in a 10-ml vial bottle using aseptic operation, freeze-dried at -20°c and the bottle was stopped with a rubber stopper to give a freeze-dried substance to be used as an injectable preparation.

#### <Example 82>

The human TPO fraction obtained in Example 56 was concentrated, subjected to aseptic filtration and filled in

a 10-ml vial bottle to give a product to be used as an injectable preparation.

### <Example 83>

The hTPO163 fraction obtained in Example 65 was concentrated, subjected to aseptic treatment and freeze-dried at -20 c to give a frozen product to be used as an injectable preparation.

### <Example 84>

The hTPO163 fraction obtained in Example 65 was concentrated, 5 ml of it was filled in a 10-ml vial bottle using aseptic operation, freeze-dried at -20°c and the bottle was sealed with a rubber stopper to give a freeze-dried product to be used as an injectable preparation.

# <Example 85>

The hTPO163 fraction obtained in Example 65 was concentrated, subjected to aseptic filtration and filled in a 10-ml vial bottle to give a product to be used as an injectable preparation.

#### <Example 86>

The human TPO fraction obtained in Example 57 was concentrated, subjected to an aseptic treatment and frozen at -20°c to give a frozen product to be used as an injectable preparation.

#### <Example 87>

The human TPO fraction obtained in Example 57 was concentrated, 5 ml of it was filled in a 10-ml vial bottle using aseptic operation, freeze-dried at -20 c and the bottle was sealed with a rubber stopper to give a freeze-dried product to be used as an injectable preparation.

#### <Example 88>

The human TPO fraction obtained in Example 57 was concentrated, subjected to an aseptic filtration an filled in a 10-ml vial bottle to give a product to be used as an injectable preparation.

#### <Example 89>

#### Aplastic Canine Plasma

Heparinized aplastic canine plasma ("APK9") or normal canine plasma ("NK9") was produced as described [Mazur, E. and South, K. Exp. Hematol. 13:1164-1172 (1985); Arriaga, M., South K., Cohen J.L. and Mazur, E.M. Blood 69: 486-492 (1987); Mazur, E., Basilico, D., Newton, J.L., Cohen, J.L., Charland, C., Sohl, P.A., and Narendran, A. Blood 76: 1771-1782 (1990)] except that 450 rads of total body irradiation were delivered to recipients.

#### <Example 90>

# Human Megakarvocvte Assav

Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely recognized manuals of molecular biology such as, for example, Sambrook, et al., (Eds), Molecular Cloning, Second Edition, ColdSpring Harbor Laboratory Press (1987) and in Ausubel, et al., (Eds), Current Protocols in Molecular Biology, Green associates/Wiley Interscience, New York (1990).

APK9 and fractionated APK9 were assayed for the ability to stimulate development of human megakaryocytes from CD34+ progenitor cells. CD34- selected cells were obtained from peripheral blood cells as described (Hokom, M.H., Choi, E., Nichol, J.L., Hornkohl, A., Arakawa, T., and Hunt, P. Molecular Biology of Haematopiesis 3:15-31,1994) and were

incubated in the following culture medium: Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 1% Glutamine Pen-strep (Irvine Scientific, Santa Ana, CA) and 10% heparinized, platelet-poor, human AB Also included were 2-mercaptoethanol (10<sup>-4</sup> M), pyruvic acid (110 µg/ml), cholesterol (7.8 µg/ml), adenosine, guanine, cytidine, uridine, thymidine, 2-deoxycytosine, 2deoxyadenosine, 2-deoxyguanosine (10 µg/ml each, Sigma); human recombinant insulin (10 μg/ml), human transferrin (300 μg/ml), soybean lipids (1%, Boehringer Mannheim, Indianapolis, IN): human recombinant basic fibroblast growth factor (2 ng/ml), Genzyme, Cambridge, MA); human recombinant epidermal growth factor (15 ng/ml), platelet-derived growth factor (10 ng/ml, Amgen, Inc., Thousand Oaks, CA). CD34selected cells were plated at 2x10<sup>5</sup> ml) culture medium, 15 µl final volume, in wells of Terasaki-style microtiter plates (Vanguard, Inc., Neptune, NJ). Cells were incubated at 37°c for 8 days in humidified boxes in 5% CO2 in air, fixed directly to the culture wells with 1% glutaraldehyde, and incubated with a monoclonal antibody cocktail (anti-GPIb, anti-GPIIB, (Biodesign) and anti-GPIb (Dako, Carpinteria, CA). The immune reaction was developed with а streptavidin-betagalactosidase detection system (HistoMark, Kirgegaard and Megakaryocytes, identified by a blue color, were counted with an inverted phase microscope at 100X magnification. Results were presented as the average number of megakaryocytes per well +/- standard error of the mean In some cases, data were presented in terms of "megakaryocyte units/ml" where the degree to which a given sample induced megakaryocyte development was normalized to the positive APK9 control for that experiment. One unit is defined as the amount of material that results in the same number of megakaryocytes as 1 µl of APK9 standard. Activity

was accepted as due to MPL ligand if it could be blocked with 5-10 µg/ml MPL-X (soluble Mpl receptor).

APK9 has been demonstrated to contain factor(s) that stimulate human megakaryocyte development in this system. CD34-selected cells incubated with 10% NK9 for 8 days show a negligible number of blue-stained megakaryocytes, whereas CD34-selected cells incubated with 10% APK9 for 8 days show a very large number of blue-stained megakaryocytes.

Figure 32 shows that increasing concentrations of Mpl-X added to the human megakaryocyte culture system increasingly block megakaryocyte development. At concentrations of Mpl-X greater than 5 μg/ml, inhibition is complete. In this experiment, CD34-selected cells were stimulated with 5% APK9. This demonstrates that an activity which interacts with Mpl-X (presumptive Mpl ligand) is necessary for human megakaryocyte development, and implies that the Mpl ligand is present in APK9 itself.

. It has been further demonstrated herein that the Mpl ligand activity necessary for human megakaryocyte development is present in APK9. APK9 (135 ml) was diluted 6fold into Iscove's media and applied to an Mpl-X affinity Unbound material (flow through was collected and concentrated to the original volume before assay. material was eluted in 10 ml of 1 M NaCl, and 20% of the pool was diafilitered and concentrated 4-fold for assay. selected cells incubated in media alone did not develop into megakaryocytes. Cells incubated in 5% APK9 (same pool as applied to column) developed into 48 +/- 8 megakaryocytes per well. Cells incubated in 10% of the unbound material did not develop into megakaryocytes. Cells incubated in 10% of the elution pool developed into 120 +/- 44 megakaryocytes per Both the column load and the elution pool activities well.

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were substantially completely inhibited with 5  $\mu$ g/ml Mpl-X in the assay.

<Example 91>

Transfection of murine or human Mpl receptor into a murine cell line

# A. Murine Mpl Receptor

The full length murine Mpl receptor cDNA was an expression vector containing subcloned into transcriptional promoter derived from the LTR of Moloney Murine Sarcome virus. 5 µg of this construct and 1 µg of the selectable marker plasmid pWLNeo (Stratagene) were coelectroporated into an IL-3 dependent murine cell line (32D. cline 23; Greenberger et al., PNAS 80:2931-2936 (1983)). Cells were cultured for 5 days to recover from the procedure, then incubated in selection media including 800 µg/ml Geneticin (G418, Sigma) and 1 ng/ml murine IL-3. surviving cells were then divided into pools of 2x10<sup>5</sup> cells and cultured until a population grew out which could be further analyzed. Six populations were tested for surface expression of Mpl receptor by FACS analysis using a polyclonal rabbit antipeptide serum. One population was chosen for FACS sorting using the same antipeptide serum as before. Singlecells clones of the parent cell line were selected by growth in 10% APK9 and Geneticin. After selection in APK9 for 35 days, the cells were maintained in 1 ng/ml murine IL-3. One of the subclones, 1A6.1, was used for this body of work.

#### B. Human Mol Receptor

The full length human Mpl receptor sequence (Vigon, I., et al., PNAS 89: 5640-5644 (1992)) was subcloned into an expression vector containing the transcriptional promoter of Maloney Murine Sarcoma virus (same vector as with the murine receptor). Six μg of this construct and 6 μg

of an amphotrophic retroviral packaging construct (Landau, N.R., Littman, D.R., *J. Virology* 66: 5110-5113 (1992)) were transfected into 3 x 10<sup>6</sup> 293 cells using a CaPO<sub>4</sub> mannalian transfection kit Stratagene. The same cells were retransfected after 2 days and again after 4 days. The day after the last transfection the 293 cells were cocultivated with the IL-3 dependent murine cell line (32D, clone 23; Greenberger et al., *PNAS* 80: 2931-2936 (1983)). After 24 hours, the 32D cells were rescued and banded in a BSA gradient (Path-o-cyte; Mills Inc.). Cells were expanded in 1 ng/ml murine IL-3 and then were selected for growth in 20% APK9. Cells were sorted for cell surface expression of receptor by FACS using a polyclonal rabbit antipeptide serum. These cells were subsequently used in the assays.

#### <Example 92>

# 1A6.1 assay for Mpl ligand

1A6.1 cells were washed free of culture IL-3 and replaced (1000 cells/15 μl total col/well) in Terasaki-style microtiter plates in alpha MEM (Gibco) supplemented with 10% fetal calf serum (FCS), Geneticin (800 μg/ml) and 1% pen/strep (Gibco) in 1:1 serial dilutions of test samples. After 48 hours, the number of viable cells per well was determined microscopically. One unit of activity was defined as that amount of activity that resulted in 200 viable cells per well. Activity was defined as due to Mpl ligand if it could be completely blocked by including 5-10 μg/ml Mpl-X in the assay. Mpl ligand activity in APK9 averaged 4400 +/- 539 units/ml of aplastic plasma. Unless otherwise indicated, units of Mpl ligand activity are defined in the 1A6.1 assay.

Assays with cells transfected with the human Mpl receptor gene were carried out in essentially the same manner as with the 1A6.1 cells.

<Example 93>
<u>Demonstration that Mol-ligand is present in aplasticplasma</u>
<u>of sera of mouse, dog, pig and human sources</u>

Mpl ligand is present in the aplastic plasma or sera from murine, canine, porcine and human sources (Table 9). Plasma was collected from BDF1 mice pre-irradiation and 12 Plasma was tested in the days post-irradiation (500 rads). 1A6.1 assay where it demonstrated 2000 units/ml activity that was substantially completely inhibitable with Mpl-X (10 Irradiated mouse plasma was also positive in the ца/ml). human megakaryocyte assay where it displayed an activity of Plasma was collected from dogs preirradiation and 10 days post-irradiation (450 rads). was tested in both the 1A6.1 assay and human megakaryocyte Activity was detected and completely inhibited by Mpl-X (10 μg/ml) in both assays. Plasma was collected from pigs pre-irradiation and 10 days post-irradiation (650 rads). Plasma was tested in both the 1A6.1 assay and the human megakaryocyte assays. In both assays it displayed Mpl ligand activity (inhibitable by 10 µg/ml Mpl-X) comparable to that found in aplastic canine plasma. Sera from aplastic humans was obtained. This material was collected from bone marrow The sera from 6 patients were transplantation patients. assayed in the 1A6.1 assay where it showed an activity of 903 units/ml, 88% of which was due to Mpl ligand (inhibitable with 10 μg/ml Mpl-X). Sera from 14 aplastic patients has also been tested in the human megakaryocyte assay. As a group, they displaced substantial activity, 941 meg units/ml, which was completely inhibitable with 10 µg/ml Mpl-X. Murine IL-3 data is included to demonstrate the specificity of the 1A6.1 assay. Although this recombinant cytokine induces growth of the cell 10 μg/ml line, it is not blocked by

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say D	+Mpl-X	<b>Q</b>	lone	0	0	0	-10		0	. Juc
uman Meg Ass (meg units/ml)	+W	0-/+0	not done	0-/+0	0-/+0	0-/+0	10+/-10	0-/+0	0-/+0	not done
Human Meg Assay (meg <u>units/ml)</u>	Media	0-/+0	1833	0-/+0	792+/-128	0-/+0	1284+/-182	0-/+0	941+/-178	not done
l A6. I Cell Assay ( <u>units/ml)</u>	X-ldW +	0+/-0	0	0-/+0	0-/+0	0-/+0	0-/+0	0-/+0	114+/-33	99-/+0009
1.46.1 Liu	Media	0-/+0	2000	0-/+0	4400+/-539	0-/+0	3866+/-1136	0-/+0	903+/-64	995-/+0009
Species		Normal mouse	Aplastic mouse	Nonnal canine	Aplistic canine	Normal porcine	Aplastic porcine	Normal human	Aplastic human	mur1L3

<Example 94>

The preparation of the human TPO derivatives expressed in E. coli system

In an attempt to improve the biological activity, stability and solubility, several TPO derivatives were designed and expressed in E. coli. The derivatives generated using the amino acid sequence set forth in SEQ ID NO:11, included [Met-2, Lys-1. Ala1. Val3. Arg129]TPO(1-163) wherein an arginine replaces a leucine at amino acid position 129 (also referred to as L129R), [Met-2, Lys-1, Ala1, Val3, Arg133]TPO(1-163) wherein an arginine replaces a histidine at amino acid position 133 (also referred to as H133R), [Met-2, Lys-1, Ala1, Val3, Arg143]TPO(1-163) wherein an arginine replaces a methionine at amino acid position 143 (also referred to as M143R), [Met2, Lys-1, Ala 1, Val3, Leu82]TPO(1-163) wherein a leucine replaces a glycine at amino acid position 82 (also referred to as G82L), [Met-2, Lys-1, Ala1, Val3, Leu146]TPO(1-163) wherein a leucine replaces a glycine at amino acid position 146 (also referred to as G146L), [Met-2, Lys-1, Ala1, Val3, Pro148)TPO(1-163) wherein a proline replaces a serine at amino acid position 148 (also referred to as S148P), [Met-2, Lys-1, Ala1, Val3, Arg<sup>59</sup>]TPO(1-163) wherein an arginine replaces a lysine at amino acid position 59 (also referred to as K59R), [Met-2, Lys-1, Ala1, Vai3, Arg115]TPO(1-163) wherein an arginine replaces a glutamate at amino acid position 115 (also referred to as Q115R).

In making the TPO derivatives, h6T (1-163), described in example 42, was used as the template, and the oligo nucleotides which have the following sequences were synthesized:

L129R: 5'-ATCTTCCGTTCTTTCCAGCACCT-3' (for the preparation of Arg-substitution derivative with Leu-129 substituted by Arg in the sequence shown in SEQ ID NO:11);

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H133R: 5'-TCTTTCCAGCGTCTGCTGCGT-3' (for the preparation of Arg-substitution derivative with His-133 substituted by Arg in the sequence shown in SEQ ID NO:11); M143R: 5'-CGTTTCCTGCGTCTGGTTGGC-3' (for the preparation of Arg-substitution derivative with Met-143 substituted by Arg in the sequence shown in SEQ ID NO:11); G82L: 5'-GGCCAGCTTCTGCCGACCTGCCT-3' (for the preparation of Leu-substitution derivative with Gly-82 substituted by Leu in the sequence shown in SEQ ID NO:11); G146L: 5-ATGCTGGTTCTGGGTTCTACCCT-3' (for the preparation of Leu-substitution derivative with Gly-146 substituted by Leu in the sequence shown in SEQ ID NO:11) S148P: 5'-GTTGGCGGTCCGACCCTGTGCG-3' (for the preparation of Pro-substitution derivative with Ser-148 substituted by Pro in the sequence shown in SEQ ID NO:11); and K59R: 5'-GAAGAGACCCGCGCTCAGGACATCC-3' (for the preparation of Arg-substitution derivative with Lys-59 substituted by Arg in the sequence shown in SEQ ID NO:11) Q115R;5'-CTGCCGCCACGTGGCCGTACCAC-3' (for the preparation of Arg-substitution derivative with Gln-115 substituted by Arg in the sequence shown in SEQ ID NO:11).

Mutant plasmids were constructed using the Sculptor in vitro mutagenesis kit (Amersham). The Xba I - Hind III fragment derived from pCFM536/h6T(1-163) described in Example 42 was introduced into Bluescript II SK(-) phagemid vector (Stratagene, California USA) after digestion with Xba I and Hind III to obtain a plasmid pSKTPO, which was then transformed into E. coli JM109. The single-stranded DNA for the mutagenesis was prepared from pSKTPO using helper phage M13KO7 (Takara shuzo, Japan). Mutations were introduced into the TPO gene according to the supplier's protocols using the oligo nucleotides as listed above. The sequencing analysis was performed with DNA sequencing kit (Applied Biosystems, USA)

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according to the supplier's protocols and the mutations of TPO were confirmed.

The Xba I - Hind III fragments prepared from mutated pSKTPO were introduced into pCFM536 digested with Xba I and Hind III. The plasmids pCFM536 carrying mutated genes were transformed into E. coli #261 in order to obtain the transformant expressing the TPO derivative genes.

The cultivation of E. coli #261 transformed with mutated pCFM 536 was performed according to Example 43 as previously described. The cell pellet of the transformant was collected and stored in the freezer for at least 1 day. The frozen cell pellet (3 g) was suspended in 30mL of 20 mM Tris buffer (pH 8.5) containing 10 mM EDTA, 10 mM DTT and 1 mM PMSF. The suspension was kept on ice and was sonicated 5 times at 1 minute (at least) intervals. The sonicated cells were collected by centrifugation at 15000 rpm for 10 min.

The pellet was suspended in 30 mL of 10 mM Tris buffer (pH 8.7) containing 8 M guanidium chloride, 5 mM EDTA, 1 mM PMSF and reduced by adding 50 mg of DTT. After stirring for 1-1.5 hour, the pH of the solution was adjusted to 5.0 by using diluted HCl and was then cooled to 4 c before dilution.

The sample solution was gradually diluted in 1.5 L of 10 mM CAPS buffer (pH 10.5) containing 30% glycerol, 3 M urea, 3 mM cystamine and 1 mM L-cysteine overnight. After allowing the sample to stir for at least 2 days, the precipitate was removed by centrifugation at 8000 rpm for 45 min. The pH of the solution was adjusted to 6.8 with 6 M phosphoric acid, and diluted two times. The solution was filtered with 2 sheets of #2 filter paper (ø=90mm, Toyo filter paper, Japan) and then loaded to the column (2.6 x 10 cm) of CM-Sepharose Fast Flow (Pharmacia). The column was washed with 400 mL of 10 mM phosphate buffer (pH 6.8) containing 15 % glycerol and 1 M of urea, and equilibrated with 10 mM phosphate buffer (pH 7.2) containing 15 % glycerol. The protein was eluted from

the column using a linear gradient system from 10 mM phosphate buffer (pH 7.2) containing 15 % glycerol to the same buffer containing 0.5 M NaCl at a flow rate of 1.0 mL/min. The protein elution was monitored by the absorbance at 280 nm and the fraction containing TPO derivative was confirmed by SDS-PAGE and collected.

After the TPO fraction (about 30 mL) was concentrated to 2 mL by using Centriprep 10 (Amicon), the mutant TPO was further purified by reversed phase HPLC (Waters) with a column of μBondasphere C4 (3.9 x 150 mm). The protein elution was performed by using a linear gradient from 0.05 % TFA in H<sub>2</sub>O to 2-propanol containing 30 % CH<sub>3</sub>CN and 0.02 % TFA with a flow rate of 0.5 mL/min for 40 min. Under this conditions, the TPO derivatives were eluted after approximately 30 min.

For the biological assay, the TPO sample thus purified was dialyzed two times against 1 L of 10 mM phosphate buffer for 2 days. After concentrating with Centriprep. 10 (Amicon) to approximately 0.1 mg/mL, the biological activity of the derivatives was evaluated by M-07e assay system as previously described. It was found that the all mutants have almost identical activity to that of h6T(1-163), the reference sample of TPO (See Figures 33 and 34).

List of deposits

Escherichia coli (pHT1-231/DH5):

FERM BP-4564 and CCTCC-M95001

Escherichia coli (pEF18S-A2a/DH5):

FERM BP-4565 and CCTCC-M95002

Escherichia coli (pHGT1/DH5):

FERM BP-4616 and CCTCC-M95003

Escherichia coli (pHTF1/DH5):

FERM BP-4617 AND CCTCC-M95004

Mouse-Mouse hybridoma P55:

FERM BP-4563 and CCTCC-C95001

CHO28/1/1/3-C6:

FERM BP-4988 and CCTCC-C95004

CHO163T-63-79-C1:

FERM BP-4989 and CCTCC-C95005

#### SEQUENCE LISTING

#### (1) GENERAL INFORMATION:

- (i) APPLICANT: MIYAZAKI, Hiroshi KATO, Takashi OHGAHI, Kinya IWAMATSU, Akihiro AKAHORI, Hiromichi KUROKI, Ryota SHIMIZU, Toshiyuki MUTO, Takanori
- (ii) TITLE OF INVENTION: PROTEIN HAVING TPO ACTIVITY
- (iii) NUMBER OF SEQUENCES: 197
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun (B) STREET: 6300 Sears Tower, 233 South Wacker Drive

  - (C) CITY: Chicago (D) STATE: Illinois
  - (E) COUNTRY: United States of America (F) ZIP: 60606-6402
- (v) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Floppy disk
   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
   (D) SOFTWARE: Patentin Release \$1.0, Version \$1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: (B) FILING DATE:

  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: JP 39090/94
  - (B) FILING DATE: 14-FEB-1994
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: JP 79842/94 (B) FILING DATE: 25-MAR-1994
- (vii) PRIOR APPLICATION DATA:
   (A) APPLICATION NUMBER: JP 155126/94
   (B) FILING DATE: 01-JUN-1994
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: JP 167328/94
    (B) FILING DATE: 15-JUN-1994
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: JP 227159/94
  - (B) FILING DATE: 17-AUG-1994
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: JP 304167/94
    (B) FILING DATE: 01-NOV-1994
- (vii) PRIOR APPLICATION DATA:
  (A) APPLICATION NUMBER: JP UNKNOWN
  (B) FILING DATE: 28-DEC-1994
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: JP 193169/94
    (B) FILING DATE: 17-AUG-1994

(VII) PRIOR APPLICATION DATA: (A) APPLICATION NUMBER: JP 298669/94 (B) FILING DATE: 01-DEC-1994	
(vii) PRIOR APPLICATION DATA: (A) APPLICATION NUMBER: JP 193916/94 (B) FILING DATE: 18-AUG-1994	
<pre>(vii) PRIOR APPLICATION DATA:     (A) APPLICATION NUMBER: US OB/212,164     (B) FILING DATE: 14-MAR-1994</pre>	
<pre>(vii) PRIOR APPLICATION DATA:     (A) APPLICATION NUMBER: US 08/221,020     (B) FILING DATE: 01-APR-1994</pre>	
<pre>(vii) PRIOR APPLICATION DATA:     (A) APPLICATION NUMBER: US 08/278,083     (B) FILING DATE: 20-JUL-1994</pre>	
<pre>(vii) PRIOR APPLICATION DATA:     (A) APPLICATION NUMBER: US 08/320,300     (B) FILING DATE: 11-OCT-1994</pre>	
<pre>(vii) PRIOR APPLICATION DATA:     (A) APPLICATION NUMBER: US 08/361,811     (B) FILING DATE: 22-DEC-1994</pre>	
<pre>(viii) ATTORNEY/AGENT INFORMATION:    (A) NAME: Borun, Michael F.    (B) REGISTRATION NUMBER: 25,447    (C) REFERENCE/DOCKET NUMBER: 01017/32425</pre>	
(ix) TELECOMMUNICATION INFORMATION: (A) TELEPHONE: 312/474-6300 (B) TELEFAX: 312/474-0448	
(2) INFORMATION FOR SEQ ID NO:1:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 261 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: double  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: cDNA to mRNA	
<ul><li>(vi) ORIGINAL SOURCE:</li><li>(A) ORGANISM: Rattus norvegicus</li><li>(H) CELL LINE: McA-RH8994</li></ul>	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:	
GGTGTACCTG GGTCCTGAAG CCCTTCTTCA CCTGGATAGA TTCCTTGGCC CACCTGTCCC	60
CACCCCACTC TGTGCAGAGG TACAAAAGCT CAAGCCGTCT CCATGGCCCC AGGAAAGATT	120
CAGGGGAGAG GCCCCACACA GGGAGCCACT GCAGTCAGAC ACCCTGGGCA GA	172
ATG GAG CTG ACT GAT TTG CTC CTG GTG GCC ATA CTT CTC CTC ACC GCA Met Glu Leu Thr Asp Leu Leu Leu Val Ala Ile Leu Leu Leu Thr Ala 1 5 15	220
AGA CTA ACT CTG TCC AGC CCA GTT CCT CCC GCC TGT GAC CC Arg Leu Thr Leu Ser Ser Pro Val Pro Pro Ala Cys Asp 20 25	261

(2)	INFORMATION	FOR	SEQ	ID	NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 147 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Leu Thr Asp Leu Leu Leu Val Ala Ile Leu Leu Leu Thr Ala

Arg Leu Thr Leu Ser Ser Pro Val Pro Pro Ala Cys Asp Pro Arg Leu
-5 10

Leu Asn Lys Leu Leu Arg Asp Ser Tyr Leu Leu His Ser Arg Leu Ser

Gln Cys Pro Asp Val Asn Pro Leu Ser Ile Pro Val Leu Leu Pro Ala 30 35 40

Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Thr Glu Gln Ser Lys 45 50 55

Ala Gln Asp Ile Leu Gly Ala Val Ser Leu Leu Leu Glu Gly Val Met
60 70 75

Ala Ala Arg Gly Gln Leu Glu Pro Ser Cys Leu Ser Ser Leu Leu Gly 80 85 90

Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Gly Leu 95 100 105

Leu Gly Thr Gln Val Ser Pro Gln Thr Tyr Arg Asn Tyr Pro Leu Thr

Gln Phe Leu 125

- (2) INFORMATION FOR SEQ ID NO:3:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 580 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: cDNA to mRNA
  - (vi) ORIGINAL SOURCE:
    - (A) ORGANISM: Homo Sapiens (F) TISSUE TYPE: liver
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTC GTG GTC ATG CTT CTC CTA ACT GCA AGG CTA ACG CTG TCC AGC CCG Leu Val Val Het Leu Leu Thr Ala Arg Leu Thr Leu Ser Ser Pro 48

GCT CCT CCT GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC 96 Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp

TCC CAT GTC CTT CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val His Pro

TTG CCT ACA CCT GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu 50 55 60 TGG AAA ACC CAG ATG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA GCA Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly Ala GTG ACC CTT CTG CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG GGA 288 Val Thr Leu Leu Clu Gly Val Het Ala Ala Arg Gly Gln Leu Gly CCC ACT TGC CTC TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC CGT 336 Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg CTC CTC CTT GGG GCC CTG CAG AGC CTC CTT GGA ACC CAG NNN NNN NNN Leu Leu Cly Ala Leu Gln Ser Leu Leu Gly Thr Gln Xaa Xaa Xaa 120 NNN NNN NNN NCC ACA GCT CAC AAG GAT CCC AAT GCC ATC TTC CTG AGC Xaa Xaa Xaa Thr Ala His Lys Asp Pro Asn Ala Ile Phe Leu Ser 130 432 TTC CAA CAC CTG CTC CGA GGA AAG GTG CGT TTC CTG ATG CTT GTA GGA 480 Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Het Leu Val Gly GGG TCC ACC CTC TGC GTC AGG CGG GCC CCA CCC ACC ACA GCT GTC CCC Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro 528 AGC AGA ACC TOT CTA GTC CTC ACA CTG AAC GAG CTC CCA AAC AGG ACT 576 Ser Arg Thr Ser Leu Val Leu Thr Leu Asn Glu Leu Pro Asn Arg Thr

#### (2) INFORMATION FOR SEQ ID NO:4:

TCT G Ser

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 253 amino acids
  - (B) TYPE: amino acid (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu Leu Thr Ala

185

580

Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val

Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser

Gin Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala 30 35 40

Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys 45 50 55

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Ala 60	Gln	Asp	Ile	Leu	Gly 65	Ala	Val	Thr	Leu	Leu 70	Leu	Glu	Gly	Val	Het 75	
Ala	Ala	Arg	Gly	Gln 80	Leu	Gly	Pro	Thr	Суя 85	Leu	Ser	Ser	Leu	<b>Le</b> u 90	Gly	
Gln	Leu	Ser	Gly 95	Gln	Val	Arg	Leu	Leu 100	Leu	Gly	Ala	Leu	Gln 105	Ser	Leu	
Leu	Gly	Thr 110	Gln	Leu	Pro	Pro	Gln 115	Gly	Arg	Thr	Thr	Ala 120	His	Lys	Asp	
Pro	Asn 125	Ala	Ile	Phe	Leu	Ser 130	Phe	Gln	His	Leu	Leu 135	Arg	Gly	Lys	Val	
Arg 140	Phe	Leu	Met	Leu	Val 145	Gly	Gly	Ser	Thr	Leu 150	Сув	Val	Arg	Arg	Ala 155	
Pro	Pro	Thr	Thr	Ala 160	Val	Pro	Ser	Arg	Thr 165	Ser	Leu	Val	Leu	Thr 170	Leu	
Asn	Glu	Leu	Pro 175		Arg	Thr	Ser	Gly 180	Leu	Leu	Glu	Thr	Asn 185	Phe	Thr	
Ala	Ser	Ala 190		Thr	Thr	Gly	Ser 195	Gly	Leu	Leu	Lys	Trp 200	Gln	Gln	CJA	
Phe	Arg 205		Lys	Ile	Pro	Gly 210	Leu	Leu	Asn	Gln	Thr 215	Ser	Arg	Ser	Leu	
Asp 220	Gln	Ile	Pro	Gly	Tyr 225	Leu	Asn	Arg	Ile	His 230		Leu				
(2)	INF	ORMA	TION	FOR	SEQ	ID	NO : 5	:								:
	(i	) SE	(A (B (C	CE C } LE } TY  ; ST	ngth Pe : Rand	: 57 nucl EDNE	6 ba eic SS:	se p acid doub								
	( <u>i</u>	i) M	OLEC	ULE	TYPE	: cD	NA t	o mis	NA.							
	( v	i) C	(A	NAL ) OR ) TI	GANI	SM:				i						
	( x	:i) S	EQUE	NCE	DESC	RIPI	: NOI	SÉÇ	ID	NO: 5	:					
AG													ACC Thr			47
TCC	CTC Lev	GAC Asp	CAP Glr	ATC	Pro	GG/ Gl <sub>y</sub>	A TAC	CTC	AAC Asi 25	ı Arç	; AT? ; Ile	A CAC	GAP Glu	Leu	TTG Leu	95
				Gly					y Pro					Le	GGA Gly	
GCC	Pro	GAG ABI 50	) Ile	TCC Sei	TC: Se:	GG Gl	A ACA	: Se	A GAG	ACI Thi	GGC Gly	C TCC y Se:	·Le	CCI Pro	A CCC Pro	191

AAC CTC CAG CCT GGA TAT TCT CCT TCC CCA ACC CAT CCT CCT ACT GGA ASN Leu Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly

239

281

	65					70					75					
CAG Gln 80	TAT Tyr	ACG Thr	CTC Leu	TTC Phe	CCT Pro 85	CTT Leu	CCA Pro	CCC Pro	ACC Thr	TTG Leu 90	CCC Pro	ACC Thr	CCT Pro	GTG Val	GTC Val 95	287
CAG Gln	CTC Leu	CAC	CCC Pro	CTG Leu 100	CTT Leu	CCT Pro	GAC Asp	CCT Pro	TCT Ser 105	GCT Ala	CCA Pro	ACG Thr	CCC Pro	ACC Thr 110	CCT Pro	335
ACC Thr	AGC Ser	CCT Pro	CTT Leu 115	CTA Leu	AAC Asn	ACA Thr	TCC Ser	TAC Tyr 120	ACC Thr	CAC His	TCC Ser	CAG Gln	AAT Asn 125	CTG Leu	TCT Ser	383
CAG Gln	GAA Glu	GGG Gly 130	TAAC	GTT	CTC A	AGACI	ACTG	CC G	ACAT	CAGC	A IIC	STCT(	CATG			432
TAC	AGCTO	CC 1	TCCC	TGC	AG GC	CGCC	CCT	GG 2	AGAC	AACT	GGA	CAAG	ATT :	CCT	ACTTTC	492
TCC	(GAA)	ACC (	CAAAC	CCC1	rg g1	LAAA?	\GGG!	A TAC	CACAC	GGAC	TGA	AAAG	GA 2	ATCAT	TTTTC	552
ACTO	TAC	ATT A	<b>LAAT</b>	CCTI	C AC	AA										576
(2)	INFO	RMAT	MOI	FOR	SEQ	ID 1	10:6	:								
	(	(i) S	(B)	LEN	GTH: PE: a		ami aci	ino a	: icid:	•						
	( i	Li) P	COLEC	ULE	TYPE	E: pz	ote	ln.								
	(>	(i) S	EQUE	ENCE	DESC	RIPT	NOI:	SEC	ID	NO: 6	<b>i</b> :					
-21	-					-15					-10					
-3	Leu				1				5					10		
Leu	Ser	Lys	Leu 15	Leu	Arg	Asp	Ser	His 20	Val	Leu	His	Ser	Arg 25	Leu	Ser	
Gln	Сув	Pro 30	Glu	Val	His	Pro	Leu 35	Pro	Thr	Pro	Val	Leu 40	Leu	Pro	Ala	
Val	Авр 45	Phe	Ser	Leu	Gly	Glu 50	Trp	Lys	Thr	Gln	Met 55	Glu	Glu	Thr	Lys	
Ala 60	Gln	yab	Ile	Leu	61y 65	Ala	Val	Thr	Leu	Leu 70	Leu	Glu	Gly	Val	Met 75	
Ala	Ala	Arg	Gly	Gln 80	Leu	Gly	Pro	Thr	Cys 85	Leu	Ser	Ser	Leu	Leu 90	Gly	
Gln	Leu	Ser	Gly 95	Gln	Val	Arg	Leu	Leu 100	Leu	Gly	Ala	Leu	Gln 105	Ser	Leu	
Leu	Gly	Thr 110	Gln	Leu	Pro	Pro	Gln 115	Gly	Arg	Thr	Thr	Ala 120	His	Lys	Asp	
Pro	Asn 125	Ala	Ile	Phe	Leu	Ser 130	Phe	Gln	His	Leu	Leu 135	λrg	Gly	Lys	Val	
Arg 140	Phe	Leu	Met	Leu	Val 145	Gly	Gly	Ser	Thr	Leu 150	Сув	Val	Arg	Arg	Ala	

Pro	Pro	Thr	Thr	Ala 160	Val	Pro	Ser	Arg	Thr 165	Ser	Leu	Val	Leu	Thr 170	Leu	•
Asn	Glu	Leu	Pro 175	Asn	Arg	Thr	Ser	Gly 180	Leu	Leu	Glu	Thr	Asn 185	Phe	Thr	
Ala	Ser	Ala 190	Arg	Thr	Thr	Gly	Ser 195	Gly	Leu	Leu	Lys	Trp 200	Gln	Gln	Gly	
Phe	Arg 205	Ala	Lys	Ile	Pro	Gly 210	Leu	Leu	Asn	Gln	Thr 215	Ser	Arg	Ser	Leu	
Asp 220	Gln	Ile	Pro	Gly	Tyr 225	Leu	Asn	Arg	Ile	His 230	Glu	Leu	Leu	Asn	Gly 235	
Thr	Arg	Gly	Leu	Phe 240	Pro	Gly	Pro	Ser	Arg 245	Arg	Thr	Leu	Gly	Ala 250	Pro	
yab	Ile	Ser	Ser 255	Cly	Thr	Ser	Asp	Thr 260	Gly	Ser	Leu	Pro	Pro 265	Asn	Leu	
Gln	Pro	Gly 270		Ser	Pro	Ser	Pro 275	Thr	His	Pro	Pro	Thr 280	Gly	Gln	Tyr	
Thr	Leu 285	Phe	Pro	Leu	Pro	Pro 290	Thr	Leu	Pro	Thr	Pro 295	Val	Val	G1n	Leu	
His 300	Pro	Leu	Leu	Pro	<b>Asp</b> 305	Pro	Ser	Ala	Pro	Thr 310	Pro	Thr	Pro	Thr	Ser 315	
Pro	Leu	Leu	Asn	Thr 320		Tyr	Thr	His	Ser 325		Asn	Leu	Ser	Gln 330	Glu	
Gly																
(2)	INF	ORMA	TION	FOR	SEQ	ID	NO: 7	:								
	(i	) SE	(B	) LE ) TY ) ST	HARA NGTH PE: RAND POLO	: 17 nucl EDNE	21 b eic S5:	ase acid doub	_	8						
	( i	.i) M	OLEC	ULE	TYPE	: cD	NA t	o mR	NA							
	(٧	·i) O	•	) OR	SOUR GANI SSUE	SM:										
	( x	:i) \$	EQUE	NCE	DESC	RIPT	ION:	SEQ	ID	NO: 7	:					
GCG	GCAC	:GAG	GGGG	GTGT	CT G	GCTG	GCGT	e 60	TCCC	TGTT	TGG	GGCC	TCT	cccc	TGAATC	50
CTI	CCTG	GGG	CCAT	GGAG	ec c	AGAC	AGAC	A CC	cccc	CCAG	1	_	lu L	TG A		113
			Lev					Leu					Lev		CTG Leu	161
TC( Se:	: Se:	Pro	GCT Ala	Pro	Pro	Ala	TG1	GAC B Asp	CTC Let	CGA Arg	y Val	CTC L Lev	AG1 Sei	Lys	CTG Leu 15	209
CT: Le:	CG:	GAG B Asi	TC Se	CAT	GT(	CT:	CAC His	E AGO	AG Ar	CTC	AGG	CAC	TGC	CC	GAG	257

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2	0	25	30	
GTT CAC CCT TTG CC Val His Pro Leu Pr 35	T ACA CCT GTC o Thr Pro Val	CTG CTG CCT GCT Leu Leu Pro Ala 40	GTG GAC TTT Val Asp Phe 45	AGC 305 Ser
TTG GGA GAA TGG AA Leu Gly Glu Trp Ly 50	A ACC CAG ATG s Thr Gln Met 55	GAG GAG ACC AAG Glu Glu Thr Lys	GCA CAG GAC Ala Gln Asp 60	ATT 353 Ile
CTG GGA GCA GTG AC Leu Gly Ala Val Th	C CTT CTG CTG r Leu Leu Leu 70	GAG GGA GTG ATG Glu Gly Val Met 75	GCA GCA CGG Ala Ala Arg	GGA 401 Gly
CAA CTG GGA CCC AC Gln Leu Gly Pro Th 80	T TGC CTC TCA r Cys Leu Ser 85	TCC CTC CTG GGG Ser Leu Leu Gly 90	CAG CTT TCT Gln Leu Ser	GGA 449 Gly 95
CAG GTC CGT CTC CT Gln Val Arg Leu Le 10	u Leu Gly Ala	CTG CAG AGC CTC Leu Gln Ser Leu 105	CTT GGA ACC Leu Gly Thr 110	CAG 497 Gln
CTT CCT CCA CAG GG Leu Pro Pro Gln Gl 115	C AGG ACC ACA y Arg Thr Thr	GCT CAC AAG GAT Ala His Lys Asp 120	CCC AAT GCC Pro Asn Ala 125	ATC 545 Ile
TTC CTG AGC TTC CA Phe Leu Ser Phe Gl 130	A CAC CTG CTC n His Leu Leu 135	CGA GGA AAG GTG Arg Gly Lys Val	CGT TTC CTG Arg Phe Leu 140	ATG 593 Met
CTT GTA GGA GGG TC Leu Val Gly Gly Se 145	C ACC CTC TGC r Thr Leu Cys 150	GTC AGG CGG GCC Val Arg Arg Ala 155	CCA CCC ACC Pro Pro Thr	ACA 641 Thr
GCT GTC CCC AGC AG Ala Val Pro Ser Ar 160	A ACC TCT CTA g Thr Ser Leu 165	GTC CTC ACA CTG Val Leu Thr Leu 170	AAC GAG CTC Asn Glu Leu	CCA 689 Pro 175
AAC AGG ACT TOT GG Asn Arg Thr Ser Gl 18	y Leu Leu Glu	ACA AAC TTC ACT Thr Asn Phe Thr 185	GCC TCA GCC Ala Ser Ala 190	AGA 737 Arg
ACA ACT GGC TCT GG Thr Thr Gly Ser Gl 195	G CTT CTG AAG y Leu Leu Lys	TGG CAG CAG GGA Trp Gln Gln Gly 200	TTC AGA GCC Phe Arg Ala 205	AAG 785 Lys
ATT CCT GGT CTG CT Ile Pro Gly Leu Le 210	G AAC CAA ACC RU Asn Gln Thr 215	Ser Arg Ser Leu	GAC CAA ATC Asp Gln Ile 220	CCC 833 Pro
GGA TAC CTG AAC AG Gly Tyr Leu Asn Ar 225	G ATA CAC GAA g Ile His Glu 230	CTC TTG AAT GGA Leu Leu Asn Gly 235	Thr Arg Gly	CTC 881 Leu
TTT CCT GGA CCC TO Phe Pro Gly Pro Se 240	A CGC AGG ACC Fr Arg Arg Thr 245	CTA GGA GCC CCG Leu Gly Ala Pro 250	GAC ATT TCC Asp Ile Ser	TCA 929 Ser 255
GGA ACA TCA GAC AC Gly Thr Ser Asp Th	ir Gly Ser Leu	CCA CCC AAC CTC Pro Pro Asn Leu 265	CAG CCT GGA Gln Pro Gly 270	TAT 977 Tyr
TCT CCT TCC CCA AC Ser Pro Ser Pro Th 275	C CAT CCT CCT THIS Pro Pro	ACT GGA CAG TAT Thr Gly Gln Tyr 280	ACG CTC TTC Thr Leu Phe 285	CCT 1025 Pro
CTT CCA CCC ACC TI	TO CCC ACC CCT	GTG GTC CAG CTC	CAC CCC CTG	CTT 1073

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Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu His Pro Leu Leu 290 295 300	
CCT GAC CCT TCT GCT CCA ACG CCC ACC CCT ACC AGC CCT CTT CTA AAC Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn 305 310	1121
ACA TCC TAC ACC CAC TCC CAG AAT CTG TCT CAG GAA GGG TAAGGTTCTC Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu Gly 320 325 330	1170
AGACACTGCC GACATCAGCA TTGTCTCGTG TACAGCTCCC TTCCCTGCAG GGCGCCCCTG	1230
GGAGACAACT GGACAAGATT TCCTACTTTC TCCTGAAACC CAAAGCCCTG GTAAAAGGGA	1290
TACACAGGAC TGAAAAGGGA ATCATTTTTC ACTGTACATT ATAAACCTTC AGAAGCTATT	1350
TTTTTAAGCT ATCAGCAATA CTCATCAGAG CAGCTAGCTC TTTGGTCTAT TTTCTGCAGA	1410
AATTTGCAAC TCACTGATTC TCTACATGCT CTTTTTCTGT GATAACTCTG CAAAGGCCTG	1470
GGCTGGCCTG GCAGTTGAAC AGAGGGAGAG ACTAACCTTG AGTCAGAAAA CAGAGAAAGG	1530
GTAATTTCCT TTGCTTCAAA TTCAAGGCCT TCCAACGCCC CCATCCCCTT TACTATCATT	1590
CTCAGTGGGA CTCTGATCCC ATATTCTTAA CAGATCTTTA CTCTTGAGAA ATGAATAAGC	1650
TTTCTCTCAG AAATGCTGTC CCTATACACT AGACAAAACT GAAAAAAAAA AAAAAAAAA	1710
AAAAAAAAA A	1721
(2) INFORMATION FOR SEQ ID NO:8:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 4506 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: double  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Genomic DNA	
<pre>(vi) ORIGINAL SOURCE:      (A) ORGANISM: Homo Sapiens      (G) CELL TYPE: peripheral leukocytes</pre>	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:	
GAATTCAGGG CTTTGGCAGT TCCAGGCTGG TCAGCATCTC AAGCCCTCCC CAGCATCTGT	60
TCACCCTGCC AGGCAGTCTC TTCCTAGAAA CTTGGTTAAA TGTTCACTCT TCTTGCTACT	120
TTCAGGATAG ATTCCTCACC CTTGGCCCGC CTTTGCCCCA CCCTACTCTG CCCAGAAGTG	180
CAAGAGCCTA AGCCGCCTCC ATGGCCCCAG GAAGGATTCA GGGGAGAGGC CCCAAACAGG	240
GAGCCACGCC AGCCAGACAC CCCGGCCAGA ATG GAG CTG ACT G GTGAGAACAC Het Glu Leu Thr -21 -20	293
ACCTGAGGGG CTAGGGCCAT ATGGAAACAT GACAGAAGGG GAGAGAGAAA GGAGACACGC	353
TGCAGGGGC AGGAAGCTGG GGGAACCCAT TCTCCCAAAA ATAAGGGGTC TGAGGGGTGG	413
ATTCCCTGGG TTTCAGGTCT GGGTCCTGAA TGGGAATTCC TGGAATACCA GCTGACAATG	473
ATTTCCTCCT CATCTTTCAA CCTCACCTCT CCTCATCTAA G AA TIG CTC CTC Glu Leu Leu	525

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-15

GTG GTC ATG CTT CTC CTA ACT GCA AGG CTA ACG CTG TCC AGC CCG GCT Val Val Het Leu Leu Thr Ala Arg Leu Thr Leu Ser Ser Pro Ala -10 -5	573
CCT CCT GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser 5 10 15	621
CAT GTC CTT CAC AGC AGA CTG GTGAGAACTC CCAACATTAT CCCCTTTATC His Val Leu His Ser Arg Leu 20 25	672
CGCGTAACTG GTAAGACACC CATACTCCCA GGAAGACACC ATCACTTCCT CTAACTCCTT	732
GACCCAATGA CTATTCTTCC CATATTGTCC CCACCTACTG ATCACACTCT CTGACAAGGA	792
TTATTCTTCA CANTACAGCC CGCATTTAAN AGCTCTCGTC TAGAGATAGT ACTCATGGAG	852
GACTAGCCTG CTTATTAGGC TACCATAGCT CTCTCTATTT CAGCTCCCTT CTCCCCCCAC	912
CAATCTTTTT CAACAG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr 30 35	<sup>.</sup> 961
CCT GTC CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr 40 45 50	1009
CAG ATG GTAAGAAAGC CATCCCTAAC CTTGGCTTCC CTAAGTCCTG TCTTCAGTTT Gln Met 55	1065
CCCACTGCTT CCCATGGATT CTCCAACATT CTTGAGCTTT TTAAAAATAT CTCACCTTCA	1125
GCTTGGCCAC CCTAACCCAA TCTACATTCA CCTATGATGA TAGCCTGTGG ATAAGATGAT	1185
GGCTTGCAGG TCCAATATGT GAATAGATTT GAAGCTGAAC ACCATGAAAA GCTGGAGAGA	1245
ARTCGCTCAT GGCCATGCCT TTGACCTATT CCTGTTCAGT CTTCTTAAAT TGGCATGAAG	1305
AAGCAAGACT CATATGTCAT CCACAGATGA CACAAAGCTG GGAAGTACCA CTAAAATAAC	1365
AAAAGACTGA ATCAAGATTC AAATCACTGA AAGACTAGGT CAAAAACAAG GTGAAACAAC	1425
AGAGATATAA ACTTCTACAT GTGGGCCGGG GGCTCACGCC TGTAATCCCA GCACTTTGGG	1485
AGGCCGAGGC AGGCAGATCA CCTGAGGGCA GGAGTTTGAG AGCAGCCTGG CCAACATGGC	1545
GAAACCCCGT CTCTACTAAG AATACAAAAT TAGCCGGGCA TGGTAGTGCA TGCCTGTAAT	1605
CCCAGCTACT TGGAAGGCTG AAGCAGGAGA ATCCCTTGAA CCCAGGAGGT GGAGGTTGTA	1665
GTGAGCTGAG ATCATGCCAA TGCACTCCAG CCTGGGTGAC AAGAGCAAAA CTCCGTCTCA	1725
AAAAGAAAAA AAAATTCTAC ATGTGTAAAT TAATGAGTAA AGTCCTATTC CAGCTTTCAG	1785
GCCACAATGC CCTGCTTCCA TCATTTAAGC CTCTGGCCCT AGCACTTCCT ACGAAAAGGA	1845
TCTGAGAGAA TTAAATTGCC CCCAAACTTA CCATGTAACA TTACTGAAGC TGCTATTCTT	1905
AAAGCTAGTA ATTCTTGTCT GTTTGATGTT TAGCATCCCC ATTGTGGAAA TGCTCGTACA	1965
GAACTCTATT CCGAGTGGAC TACACTTAAA TATACTGGCC TGAACACCGG ACATCCCCCT	2025
GAAGACATAT GCTAATTTAT TAAGAGGGAC CATATTAAAC TAACATGTGT CTAGAAAGCA	2085

	•		
GCAGCCTGAA CAGAAAGAGA CTAGAA			2145
ATCTATCCTC AAGAACCCTA GCGTCC	CTTC TTCCTTCAGG	ACTGAGTCAG GGAAGAAGGG	2205
CAGTTCCTAT GGGTCCCTTC TAGTCC	TTTC TTTTCATCCT	TATGATCATT ATGGTAGAGT	2265
CTCATACCTA CATTTAGTTT ATTTAT	TATT ATTATTTGAG	ACGGAGTCTC ACTCTATCCC	2325
CCAGGCTGGA GTGCAGTGGC ATGATC	TCAR CTCACTGCAR	CCTCAGCCTC CCGGATTCAA	2385
GCGATTCTCC TGCCTCAGTC TCCCAA	GTAG CTGGGATTAC	AGGTGCCCAC CGCCATGCCC	2445
AGCTAATTTT TGTATTTTTG GTAGAG	ATGG GGTTTCACCA	TGTTGGCCAG GCTGATCTTG	2505
AACTCCTGAC CTCAGGTGAT CCACCT	GCCT CAGCCTCCCA	AAGTGCTGGG ATTACAGGCG	2565
TGAGCCACTG CACCCAGCCT TCATTC	AGTT TAAAAATCAA	ATGATCCTAA GGTTTTGCAG	2625
CAGAAAGAGT AAATTTGCAG CACTAG	AACC AAGAGGTAAA	AGCTGTAACA GGGCAGATTT	2685
CAGCAACGTA AGAAAAAAGG AGCTCT	TCTC ACTGAAACCA	AGTGTAAGAC CAGGCTGGAC	2745
TAGAGGACAC GGGAGTTTTT GAAGCA	GAGG CTGATGACCA	GCTGTCGGGA GACTGTGAAG	2805
GAATTCCTGC CCTGGGTGGG ACCTTG	GTCC TGTCCAGTTC	TCAGCCTGTA TGATTCACTC	2865
TGCTGGCTAC TCCTAAGGCT CCCCAC	CCGC TTTTAGTGTG	CCCTTTGAGG CAGTGCGCTT	2925
	Glu Thr Lys Ala	Gln Asp Ile Leu Gly 65	2977
GCA GTG ACC CTT CTG CTG GAG ( Ala Val Thr Leu Leu Leu Glu ( 70	GGA GTG ATG GCA Gly Val Het Ala 75	GCA CGG GGA CAA CTG Ala Arg Gly Gln Leu 80	3025
GGA CCC ACT TGC CTC TCA TCC ( Gly Pro Thr Cys Leu Ser Ser 1 85	CTC CTG GGG CAG Leu Leu Gly Gln 90	CTT TCT GGA CAG GTC Leu Ser Gly Gln Val 95	3073
CGT CTC CTC CTT GGG GCC CTG ( Arg Leu Leu Gly Ala Leu ( 100	CAG AGC CTC CTT Gln Ser Leu Leu 105	GGA ACC CAG Gly Thr Gln 110	3115
STAAGTCCCC AGTCAAGGGA TCTGTAG	GAAA CTGTTCTTTT	CTGACTCAGT CCCCCTAGAA	3175
GACCTGAGGG AAGAAGGGCT CTTCCAG	GGA CCTCAAGGGC	AGAAGAGCTG ATCTACTAAG	3235
AGTGCTCCCT GCCAGCCACA ATGCCTC	GGGT ACTGGCATCC	TGTCTTTCCT ACTTAGACAA	3295
GGAGGCCTG AGATCTGGCC CTGGTG	TTTG GCCTCAGGAC	CATCCTCTGC CCTCAG	3351
CTT CCT CCA CAG GGC AGG ACC A Leu Pro Pro Gin Gly Arg Thr 1 115	ACA GCT CAC AAG Thr Ala His Lys 120	GAT CCC AAT GCC ATC Asp Pro Asn Ala Ile 125	3399
TTC CTG AGC TTC CAA CAC CTG C Phe Leu Ser Phe Gln His Leu I 130	CTC CGA GGA AAG Leu Arg Gly Lys 135	GTG CGT TTC CTG ATG Val Arg Phe Leu Met 140	3447
CTT GTA GGA GGG TCC ACC CTC 1 Leu Val Gly Gly Ser Thr Leu C 145	TGC GTC AGG CGG Cys Val Arg Arg	GCC CCA CCC ACC ACA Ala Pro Pro Thr Thr 155	3495
SCT GTC CCC AGC AGA ACC TCT CALLS Val Pro Ser Arg Thr Ser I 165	CTA GTC CTC ACA Leu Val Leu Thr 170	CTG AAC GAG CTC CCA Leu Asn Glu Leu Pro 175	3543

AAC Aan	agg Arg	ACT Thr	TCT Ser	GGA Gly 180	TTG Leu	TTG Leu	GAG Glu	ACA Thr	AAC Asn 185	TTC Phe	ACT Thr	GCC Ala	TCA Ser	GCC Ala 190	AGA <sup>.</sup>	3591
ACT Thr	ACT Thr	Gly	TCT Ser 195	GJ Y GGG	CTT Leu	CTG Leu	AAG Lys	TGG Trp 200	CAG Gln	CAG Gln	GGA Gly	TTC Phe	AGA Arg 205	GCC Ala	AAG Lys	3639
ATT Ile	CCT Pro	GGT Gly 210	CTG Leu	CTG Leu	AAC Asn	CAA Gln	ACC Thr 215	TCC Ser	AGĞ Arg	TCC Ser	CTG Leu	GAC Asp 220	CAA Gln	ATC Ile	CCC Pro	3687
GGA Gly	TAC Tyr 225	CTG Leu	AAC Ann	AGG Arg	ATA Ile	CAC His 230	GAA Glu	CTC Leu	TTG Leu	AAT Asn	GGA Gly 235	ACT Thr	CGT	GGA Gly	CTC Leu	3735
														TCC Ser		3783
														GGA Gly 270		3831
														TTC Phe	CCT Pro	3879
			Thr												CTT Leu	3927
															AAC Asn	3975
	Ser										Glu			GGTT	CTC	4024
AGA	CACT	GCC	GACA	TCAG	CA T	TGTC	TCAT	S TA	CAGC	TCCC	TIC	CCTG	CAG	GGCG	CCCCTG	4084
GGA	GACA	ACT	GGAC	AAGA	TT T	CCTA	CTTT	C TC	CTGA	AACC	CAA	AGCC	CTG	GTAA	AAGGGA	4144
TAC	ACAG	GAC	TGAA	AAGG	GA A	TCAT	TTTT	C AC	TGTA	CATT	ATA	AACC	TTC	AGAA	GCTATT	4204
TTT	TTAA	CCT	ATCA	GCAA	TA C	TCAT	CAGA	G CA	GCTA	GCTC	TTT	GGTC	TAT	TTTC	TGCAGA	4264
AAT	TTGC	AAC	TCAC	TGAT	TC T	CTAC	ATGC	T CT	TTTT	CTGT	GAT	AACT	CTG	CAAA	GGCCTG	4324
GGC	TGGC	CTG	GCAG	TTGA	AC A	GAGG	GAGA	G AC	TAAC	CTTG	AGT	CAGA	AAA	CAGA	GAAAGG	4384
GTA	ATTT	CCT	TTGC	TTCA	I AA	TCAA	GGCC	T TC	CAAC	GCCC	CCA	TCCC	CTT	TACT	ATCATI	4444
CIC	AGTG	GGA	CTCI	GATO	CC A	TATT	CTTA	A CA	GATC	TTTA	CŢC	TTGA	GAA	ATGA	ATAAGO	4504
TT																4506

# (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:

  (A) LENGTH: 416 base pairs

  (B) TYPE: nucleic acid

  (C) STRANDEDNESS: double

  (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: synthetic DNA

CTAGAAAAAA	CCAAGGAGGT	AATAAATAAT	GAGCCCGGCT	CCGCCAGCTT	GTGACCTTCG	60
TGTTCTGTCT	AAACTGCTTC	GCGACTCTCA	CGTGCTGCAC	TCTCGTCTGT	CCCAGTGCCC	120
GGAAGTTCAC	CCGCTGCCGA	CCCCGGTTCT	GCTTCCGGCT	GTCGACTTCT	CCCTGGGTGA	180
ATGGAAAACC	CAGATGGAAG	AGACCAAAGC	TCAGGACATC	CTGGGTGCAG	TAACTCTGCT	240
TCTGGAAGGC	GTTATGGCTG	CACGTGGCCA	GCTTGGCCCG	ACCTGCCTGT	CTTCCCTGCT	300
TGGCCAGCTG	TCTGGCCAGG	TTCGTCTGCT	GCTCGGCGCT	CTGCAGTCTC	TGCTTGGCAC	360
CCAGCTGCCG	CCACAGGGCC	GTACCACTGC	TCACAAGGAT	CCGAACGCTA	TCTTCC	416

#### (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 179 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Val Pro Arg Gly Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val

Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser 15 20 25

Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala 30 35 40

Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys 45 50 55

Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met 60 70 75

Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly 80 85 90

Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu 95 100 105

Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp 110 115 120

Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val 125 130 135

Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala 145 150 155

Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu 165

Asn Glu Leu

- (2) INFORMATION FOR SEQ ID NO:11:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 535 base pairs (B) TYPE: nucleic acid

(C) STRANDEDNESS: double (D) TOPOLOGY: linear

	(ii	L) MC	LECT	ILE 1	YPE:	syr	thet	ic D	ANC							
	(vi	L) OF					omo	Sapi	iens							
	(x)	L) SE	QUEN	CE D	ESC	RIPTI	ON:	SEQ	ID 1	NO:1	1:					
CTA	GAAAJ	VAA (	CAAC	GAGG	ST AJ	LAAT	L ATA	ATG 2 Het 1 -2	AAA (	GCA ( Ala 1	CCT (	TA (	CCA ( Pro 1	CCT (Pro 1	CA Ala	52
Cys	GAT Asp	TTA Leu	CGG Arg 10	GTC Val	CTG Leu	TCT Ser	AAA Lys	CTG Leu 15	CTG Leu	CGC Arg	GAC Asp	TCT Ser	CAC His 20	GTG Val	CTG Leu	100
CAC His	TCT Ser	CGT Arg 25	CTG Leu	TCC Ser	CAG Gln	TGC	CCG Pro 30	GAA Glu	GTT Val	CAC His	CCG Pro	CTG Leu 35	CCG Pro	ACC Thr	CCG Pro	148
GTT Val	CTG Leu 40	CTT Leu	CCG Pro	GCT Ala	GTC Val	GAC Asp 45	TTC Phe	TCC Ser	CTG Leu	Gly	GAA Glu 50	TGG Trp	AAA Lys	ACC Thr	CAG Gln	196
ATG Met 55	GAA Glu	GAG Glu	ACC Thr	AAA Lys	GCT Ala 60	CAG Gln	GAC Asp	ATC	CTG Leu	GGT Gly 65	GCA Ala	GTA Val	ACT Thr	CTG Leu	CTT Leu 70	244
CTG Leu	GAA Glu	GCC	GTT Val	ATG Met 75	GCT Ala	GCA Ala	CGT Arg	ely eec	CAG Gln 80	CTT Leu	GGC Gly	CCG Pro	ACC Thr	TGC Cys 85	CTG Leu	292
ICT Ser	TCC Ser	CTG Leu	CTT Leu 90	ely ecc	CAG Gln	CTG Leu	TCT Ser	GGC Gly 95	CAG Gln	GTT Val	CGT Arg	CTG Leu	CTG Leu 100	CTC Leu	GGC	340
SCT Ala	CTG	CAG Gln 105	TCT Ser	CTG Leu	CTT Leu	GGC Gly	ACC Thr 110	CAG Gln	CTG Leu	CCG Pro	CCA Pro	CAG Gln 115	GGC	CGT Arg	ACC Thr	388
ACT The	GCT Ala 120	CAC His	AAG Lys	GAT	CCG Pro	AAC Asn 125	GCT Ala	ATC Ile	TTC Phe	CTG Leu	TCT Ser 130	TTC Phe	CAG Gln	CAC His	CTG Leu	436
CTG Leu 135	CGT	GGC Gly	AAA Lys	GTT Val	CGT Arg 140	TTC Phe	CTG Leu	ATG Met	CTG Leu	GTT Val 145	GCC	GGT Gly	TCT Ser	ACC Thr	CTG Leu 150	484
TGC Cys	GTT Val	CGT Arg	CGG Arg	GCG Ala 155	CCG Pro	CCA Pro	ACC Thr	ACT Thr	GCT Ala 160	Val	CCG Pro	TCT Ser	TAA	TGAA	AGC	533
TT																535

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:

  (A) LENGTH: 535 base pairs

  (B) TYPE: nucleic acid

  (C) STRANDEDNESS: double

  (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: synthetic DNA
- (VI) ORIGINAL SOURCE:

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#### (A) ORGANISM: Homo Sapiens

(xi)	SEQUENCE	DESCRIPTION:	SEQ	ID	NO:12:	
------	----------	--------------	-----	----	--------	--

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:																	
CTAGAAAAAA CCAAGGAGGT AATAAATA ATG AAA TCT CCT GCA CCA CCT GCA Het Lys Ser Pro Als Pro Pro Als -2 1 5														;	52		
										CGC						1	00
										CAC His						1	48
										GGT Gly						1	96
ATG Met 55	GAA Glu	GAG Glu	ACC Thr	AAA Lys	GCT Ala 60	CAG Gln	GAC Asp	ATC Ile	CTG Leu	GGT Gly 65	GCA Ala	GTA Val	ACT Thr	CTG Leu	CTT Leu 70		44
										CTT Leu						2	92
										GTT Val						3	40
										CCG Pro						3	88
		His								CTG Leu						4	36
						Phe				GTT Val 145						4	84
					Pro					GTT Val				TGAA	AGC	5	33
TT																5	35
(2)	INF	ORMA	TION	FOR	SEQ	ID	NO:1	3:									•
		.) SE	(A (B (C) (D)	) LE ) TY ) SI ) TO	NGTH PE: RAND POLC	: 55 nucl EDNE GY:	5 ba eic SS: line	se p acid doub ar	le	:							
	12	. <u>-</u> ) M	اعدن	عرب.	IIPE		MM T	.o mu	TAN.								

- (x1) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATG GAG CTG ACT GAA TTG CTC CTC GTG GTC ATG CTT CTC CTA ACT GCA

	Glu -20	Leu	Thr	Glu	Leu	Leu -15	Leu	Val	Val	Xet	Leu -10	Leu	Leu	Thr	Ala	
			CTG Leu													96
CTC Leu	AGT Ser	AAA Lys	CTG Leu 15	CTT Leu	CGT Arg	GAC Asp	TCC Ser	CAT His 20	GTC Val	CTT Leu	CAC His	AGC Ser	AGA Arg 25	CTG Leu	AGC Ser	144
			GAG Glu													192
			AGC Ser													240
GCA Ala 60	CAG Gln	GAC Asp	ATT Ile	CTG Leu	GGA Gly 65	GCA Ala	GTG Val	ACC Thr	CTT Leu	CTG Leu 70	CTG Leu	GAG Glu	GGA Gly	GTG Val	ATG Met 75	288
			GGA Gly													336
			GGA Gly 95											Ser	CTC Leu	384
													His		GAT Asp	432
		Ala					Phe					Arg			GTG Val	480
	Phe					Gly					Cys				GCC Ala 155	528
			ACA Thr		Val				•							555
(2)	INF	ORKA	TION	FOR	SEC	ID	NO: 1	4: -								
	(i	.) SE	( E	) LE 3) TY 3) ST	ngti Pe: Rani	CTER 1: 10 nucl EDNE CGY:	43 b eic SS:	ase acid doub	l	rs						
	( i	.i) 1	COLEC	CULE	TYPE	E: 83	nthe	tic	DNA							•
	(1	/i) (	ORIGI			RCE:	Homo	Sap	pien							
	()	(i) :	SEQUI	ENCE	DES	CRIP:	CION	SE	2 ID	NO:	14:					
CT	AGAAJ	<b>AAAA</b>	CCAI	AGGA	GGT :	aatai	AATA		Lys						GCA Ala	52
TG	T GA	T TT	A CG	G GT	c cī	G TC	T AA	A CT	G CT	G CG	C GA	c TC	T CA	C GT	CTG	100

Сув	yab	Leu	Arg 10	Val	Leu	Ser	Lys	L <b>e</b> u 15	Leu	<b>λ</b> rg	Asp	Ser	His 20	Val	Leu	
					CAG Gln											148
					GTC Val											196
ATG Het 55	GAA Glu	GAG Glu	ACC Thr	AAA Lys	GCT Ala 60	CAG Gln	GAC Asp	ATC Ile	CTG Leu	GGT Gly 65	GCA Ala	GTA Val	ACT Thr	CTG Leu	CTT Leu 70	244
CTG Leu	GAA Glu	GGC Gly	GTT Val	ATG Met 75	GCT Ala	GCA Ala	CGT Arg	GGC Gly	CAG Gln 80	CTT Leu	ej eec	CCG Pro	ACC Thr	TGC Cys 85	CTG Leu	292
TCT Ser	TCC Ser	· CTG Leu	CTT Leu 90	GGC	CAG Gln	CTG Leu	TCT Ser	GGC Gly 95	CAG Gln	GTT Val	CGT Arg	CTG Leu	CTG Leu 100	CTC	GC	340
					CTT											388
					CCG Pro											436
	Arg				CGT Arg 140											484
					CCG Pro											532
				Leu	AAC Asn				Asn							580
			Phe					Arg					Gly		CTG Leu	628
		Gln					Ala					Leu			CAG Gln	676
	Ser					Gln					Leu				CAT His 230	724
GAA Glu	CTG Leu	CTG Leu	AAC Asn	GGC Gly 235	The	CGT	GGC Gly	CTC Leu	Phe 240	Pro	GGC	CCG Pro	AGC Ser	CGT Arg 245	CGC	772
ACC Thr	CTC Leu	GCC	GCG Ala 250	Pro	GAI Asp	ATC	AGC Ser	Ser 255	Gly	Thi	AGC Ser	GAT	ACC Thr 260	Gly	AGC Ser	820
CTC	Pro	Pro 265	) Asr	CTC	CAC Glr	CCG Pro	GGC Gly 270	Ty	AGC Ser	Pro	AGC Ser	Pro 275	Thr	CAT His	CCG Pro	868

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									CTG Leu							916
CCG Pro 295	GTG Val	GTT Val	CAG Gln	CTG Leu	CAT His 300	CCG Pro	CTG Leu	CTG Leu	CCG Pro	GAT Asp 305	CCG Pro	AGC Ser	GCG Ala	CCG Pro	ACC Thr 310	964
									ACC Thr 320							1012
					GJY GGC		rgaa(	GCT :	IGA			•				1043
(2)	INF	AMAC	TION	FOR	SEQ	וםו	NO:1	5:			•					
	(غ	, i) M	(A (B (C (D	) LE ) TY ) ST: ) TO		: 45 nucle EDNE GY: : Ot	bas sic : SS: : line	e pa acid sing ar nucl			5.					
	•	•						_								
									TTTT	TTTT	TTT	TT			45	
(2)					SEQ											
	(1	) SE	A) E) C)	) LE ) TY :) ST	HARA NGTH PE: RAND POLC	: 13 nucl EDNE	bas eic S5:	e pa acid sing								
	(i	i) M	OLEC	ULE	TYPE	: 05	her	nucl	eic	acid						
	( x	i) 5	EQUE	NCE	DESC	RIPT	ION:	SEQ	ID	NO: 1	6:					
AAT	TCGG	CAC	GAG												13	
(2)	INF	ORMA	TION	FOF	SEÇ	ID	NO: 1	7:								
	į)	.) SE	- () (E	() LE () TY () S1	HARA INGTH (PE: TRANI OPOLO	i: 9 nucl	base eic SS:	pai acid sinc	ı .							
	( i	i) P	HOLE	CULE	TYPE	: Ot	her	nucl	leic	acid	1					
	()	i) s	SEQUI	ENCE	DES	CRIP	CION:	SE(	םו מ	NO: 3	17:					
CTC	GTG	CCG													9	
(2)	IN	FORM	ATIO	N FO	R SE	םו ב	NO:	18:								
	( :	i) Si	(1	A) L: B) T' C) S'	CHAR ENGTI YPE: TRAN	H: 1: ami: DEDN:	2 am. no a: ESS:	ino a	acid	8						

(ii) MOLECULE TYPE: peptide

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
- Ile Pro Val Pro Pro Ala Cys Asp Pro Arg Leu Leu
- (2) INFORMATION FOR SEQ ID NO:19:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 12 amino acids
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Thr Pro Val Pro Pro Ala Cys Asp Pro Arg Leu Leu

- (2) INFORMATION FOR SEQ ID NO:20:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 12 amino acids
    - (B) TYPE: amino acid (C) STRANDEDNESS:

    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ser Pro Val Pro Pro Ala Cys Asp Pro Arg Leu Leu

- (2) INFORMATION FOR SEQ ID NO:21:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 17 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: Other nucleic acid
  - (ix) FEATURE:
    - (A) NAME/KEY: modified\_base (B) LOCATION: 12 & 15

    - (D) OTHER INFORMATION: /mod base= i
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

### ACGCTGGGGG CNGANGA

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: Other nucleic acid
- (ix) FEATURE:
  - (A) NAME/KEY: modified\_base
  - (B) LOCATION: 12 & 15

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(D) OTHER INFORMATION: /mod_base= i	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:	
ACACTAGGAT CNAANAA	17
(2) INFORMATION FOR SEQ ID NO:23:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 17 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(ix) FEATURE:  (Ā) ÑĀME/KĒY: modified_base  (B) LOCATION: 12 & 15  (D) OTHER INFORMATION: /mod_base= i	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:	
ACGCTGGGTG CNGANGA	17
(2) INFORMATION FOR SEQ ID NO:24:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 17 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(ix) FEATURE:  (A) NAME/KEY: modified_base  (B) LOCATION: 12 & 15  (D) OTHER INFORMATION: /mod_base= i	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:	
ACGCTGGGCG CNGANGA	17
(2) INFORMATION FOR SEQ ID NO:25:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 17 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
.(x1) SEQUENCE DESCRIPTION: SEQ ID NO:25:	
GGGGGGCGGA CGCTGGG	17
(2) INFORMATION FOR SEQ ID NO:26:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 17 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGAGGACGAA CACTAGG	17
(2) INFORMATION FOR SEQ ID NO:27:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 17 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:	
GGTGGTCGTA CGCTGGG	17
(2) INFORMATION FOR SEQ ID NO:28:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 17 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:	
GGCGGCCGCA CGCTGGG	17
(2) INFORMATION FOR SEQ ID NO:29:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:	
GGATCTTGGT TCATTCTCAA G	21
(2) INFORMATION FOR SEQ ID NO:30:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 20 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:	
CCTCAGACAG TGGTTCAAAG	20
(2) INFORMATION FOR SEQ ID NO:31:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:	
CGAGGGTGTA CCTGGGTCCT G	21
(2) INFORMATION FOR SEQ ID NO:32:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:	
CAGAGTTAGT CTTGCGGTGA G	21
(2) INFORMATION FOR SEQ ID NO:33:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:	
CCTGTCCTGC TGCCTGCTGT G	21
(2) INFORMATION FOR SEQ ID NO:34:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) HOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:	
TGAAGTTCGT CTCCAACAAT C	21
(2) INFORMATION FOR SEQ ID NO:35:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid.	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:	
ATGGAGCTGA CTGATTTGCT C	21
(2) INFORMATION FOR SEQ ID NO:36;	
<ul><li>(i) SEQUENCE CHARACTERISTICS:</li><li>(A) LENGTH: 21 base pairs</li><li>(B) TYPE: nucleic acid</li></ul>	

(C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:	
TGAAGTTCGT CTCCAACAAT C	21
(2) INFORMATION FOR SEQ ID NO:37:	•
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:	
TTGTGACCTC CGAGTCCTCA G	21
(2) INFORMATION FOR SEQ ID NO:38:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:	
TGACGCAGAG GGTGGACCCT C	21
(2) INFORMATION FOR SEQ ID NO:39:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:	
AGCAGAACCT CTCTAGTCCT C	21
(2) INFORMATION FOR SEQ ID NO:40:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:	
ACACTGAACG AGCTCCCAAA C	21
(2) INFORMATION FOR SEQ ID NO:41:	

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(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 base pairs	
(B) TYPE: nucleic acid (C) STRANDEDNESS: single	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:	
AACTACTGGC TCTGGGCTTC T	21
(2) INFORMATION FOR SEQ ID NO:42:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:	
AGGGATTCAG AGCCAAGATT C	21
(2) INFORMATION FOR SEQ ID NO:43:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 31 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:	
TAGCGGCCGC TTTTTTTTT TTTTTTTGGG G	31
(2) INFORMATION FOR SEQ ID NO:44:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 31 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:	
TAGCGGCCGC TITTTTTTTT TTTTTTAAA A	31
(2) INFORMATION FOR SEQ ID NO:45:	31
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 31 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:	
TAGCGGCCGC TITTTTTTTT TTTTTTTCTT T	31

(2)	INFORMATION FOR SEQ ID NO:46:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 31 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:	
TAGO	GGCCGC TTTTTTTTT TTTTTTGCC C	31
(2)	INFORMATION FOR SEQ ID NO:47:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH:,21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:	
TAGO	EGGCCGC TTTTTTTTT T	21
(2)	INFORMATION FOR SEQ ID NO:48:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
•	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:	
TTGI	CGACCTC CGAGTCCTCA G	21
(2)	INFORMATION FOR SEQ ID NO:49:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:	
AGGA	ATGGGTT GGGGAAGGAG A	-21
(2)	INFORMATION FOR SEQ ID NO:50:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TTGTGACCTC CGAGTCCTCA G	21
(2) INFORMATION FOR SEQ ID NO:51:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:	
AGGGAAGAGC GTATACTGTC C	21
(2) INFORMATION FOR SEQ ID NO:52:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:	
GGCCAGCCAG ACACCCCGGC C	21
(2) INFORMATION FOR SEQ ID NO:53:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:	
ATGGGAGTCA CGAAGCAGTT T	21
(2) INFORMATION FOR SEQ ID NO:54:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:	•
TGCGTTTCCT GATGCTTGTA G	21
(2) INFORMATION FOR SEQ ID NO:55:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:	
	21
(2) INFORMATION FOR SEQ ID NO:56:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 30 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:	
TTTGAATTCG GCCAGCCAGA CACCCCGGCC	30
(2) INFORMATION FOR SEQ ID NO:57:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 39 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:	
TTTGCGGCCG CTCATTAGCT GGGGACAGCT GTGGTGGGT	39
(2) INFORMATION FOR SEQ ID NO:58:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 42 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:	
TTTGCGGCCG CTCATTACAG TGTGAGGACT AGAGAGGTTC TG	42
(2) INFORMATION FOR SEQ ID NO:59:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 42 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:	
TTTGCGGCCG CTCATTATCT GGCTGAGGCA GTGAAGTTTG TC	12
(2) INFORMATION FOR SEQ ID NO:60:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 42 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:	
TTTGCGGCCG CTCATTACAG ACCAGGAATC TTGGCTCTGAA T	42
(2) INFORMATION FOR SEQ ID NO:61:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 30 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:	
TTTGAATTCG GCCAGCCAGA CACCCCGGCC	30
(2) INFORMATION FOR SEQ ID NO:62:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 41 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:	
TTTGCGGCCG CTCATTAGAG GGTGGACCCT CCTACAAGCA T	41
(2) INFORMATION FOR SEQ ID NO:63:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 40 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:	
TTTGCGGCCG CTCATTAGCA GAGGGTGGAC CCTCCTACAA	40
(2) INFORMATION FOR SEQ ID NO:64:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 38 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:	
TTTGCGGCCG CTCATTACCT GACGCAGAGG GTGGACCC	38
(2) INFORMATION FOR SEQ ID NO:65:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 38 base pairs  (B) TYPE: nucleic acid	

(C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:	
TTTGCGGCCG CTCATTACCG CCTGACGCAG AGGGTGGA	38
(2) INFORMATION FOR SEQ ID NO:66:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 38 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:	
TTTGCGGCCG CTCATTAGGC CCGCCTGACG CAGAGGGT	38
(2) INFORMATION FOR SEQ ID NO:67:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 38 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENÇE DESCRIPTION: SEQ ID NO:67:	
TTTGCGGCCG CTCATTATGG GGCCCGCCTG ACGCAGAG	38
(2) INFORMATION FOR SEQ ID NO:68:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 38 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:	
TTTGCGGCCG CTCATTAGGG TGGGGCCCGC CTGACGCA	38
(2) INFORMATION FOR SEQ ID NO:69:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 30 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:	
TTTGAATTCG GCCAGCCAGA CACCCCGGCC	30
(2) INFORMATION FOR SEO ID NO:70	

•		
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 41 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear		
(ii) MOLECULE TYPE: Other nucleic acid		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:		
TTTGCGGCCG CTCATTATTC GTGTATCCTG TTCAGGTATC C	41	
(2) INFORMATION FOR SEQ ID NO:71:		
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 39 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear		
(ii) MOLECULE TYPE: Other nucleic acid		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:		
TTTGCGGCCG CTCATTAGCT GGGGACAGCT GTGGTGGGT	39	
(2) INFORMATION FOR SEQ ID NO:72:		
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 50 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear		
(ii) HOLECULE TYPE: Other nucleic acid		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:		
AGTARACTGC TTCGTGACTC CCATGTCCTT CACAGCAGAC TGAGCCAGTG	50	
(2) INFORMATION FOR SEQ ID NO:73:		
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 49 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear		
(ii) MOLECULE TYPE: Other nucleic acid		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:		
CATGGGAGTC ACGAAGCAGT TTACTGGACA GCGTTAGCCT TGCAGTTAG	49	
(2) INFORMATION FOR SEQ ID NO:74:		
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 49 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear		
(ii) MOLECULE TYPE: Other nucleic acid	•	•
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:		
TGTGACCTCC GAGTCCTCAG TAAACTGCTT CGTGACTCCC ATGTCCTTC	49	

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(2)	INFORMATION FOR SEQ ID NO:75:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 48 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:	
TTTP	ACTGAGG ACTCGGAGGT CACAGGACAG CGTTAGCCTT GCAGTTAG	48
(2)	INFORMATION FOR SEQ ID NO:76:	
	(i) SEQUENCE CHARACTERISTICS:  {A} LENGTH: 49 base pairs  {B} TYPE: nucleic acid  (C) STRANDEDNESS: single  {D} TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:	
GAC	CTCCGAG TCCTCAGTAA ACTGCTTCGT GACTCCCATG TCCTTCACA	49
(2)	INFORMATION FOR SEQ ID NO:77:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 48 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:	
CAG	TTTACTG AGGACTCGGA GGTCGGACAG CGTTAGCCTT GCAGTTAG	48
(2)	INFORMATION FOR SEQ ID NO:78:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: Other nucleic acid	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:	
TTG	TGACCTC CGAGTCCTCA G	21
(2)	INFORMATION FOR SEQ ID NO:79:	
	(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid (x1) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CAGGTATCCG GGGATTTGGT C 21	
(2) INFORMATION FOR SEQ ID NO:80:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:	
TGCGTTTCCT GATGCTTGTA G 21	
(2) INFORMATION FOR SEQ ID NO:81:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 35 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:	
GAGAGAGCGG CCGCTTACCC TTCCTGAGAC AGATT 35	
(2) INFORMATION FOR SEQ ID NO:82:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 6 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:	
GAGAGA 6	
(2) INFORMATION FOR SEQ ID NO:83:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 70 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:	
CTAGAAAAAA CCAAGGAGGT AATAAATAAT GAGCCCGGCT CCGCCAGCTT GTGACCTTCG	60
TGTTCTGTCT	70
(2) INFORMATION FOR SEQ ID NO:84:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 72 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:	
CAGTTTAGAC AGAACACGAA GGTCACAAGC TGGCGGAGCC GGGCTCATTA TTTATTACCT	60
CCTTGGTTTT TT	72
(2) INFORMATION FOR SEQ ID NO:85:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 69 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:	
AAACTGCTTC GCGACTCTCA CGTGCTGCAC TCTCGTCTGT CCCAGTGCCC GGAAGTTCAC	60
CCGCTGCCG	69 ·
(2) INFORMATION FOR SEQ ID NO:86:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 69 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:	
EGGGGTCGGC AGCGGGTGAA CTTCCGGGCA CTGGGACAGA CGAGAGTGCA GCACGTGAGA	60
STCGCGAAG	69
(2) INFORMATION FOR SEQ ID NO:87:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 69 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:	
ACCCCGGTTC TGCTTCCGGC TGTCGACTTC TCCCTGGGTG AATGGAAAAC CCAGATGGAA	60
FAGACCAAA	69
(2) INFORMATION FOR SEQ ID NO:88:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 69 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CTGAGCTTTG GTCTCTCCA TCTGGGTTTT CCATTCACCC AGGGAGAAGT CGACAGCCGG	60
AAGCAGAAC	69
(2) INFORMATION FOR SEQ ID NO:89:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 69 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:	
GCTCAGGACA TCCTGGGTGC AGTAACTCTG CTTCTGGAAG GCGTTATGGC TGCACGTGGC	60
CAGCTTGGC	69
(2) INFORMATION FOR SEQ ID NO:90:	0,
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 69 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:	
GGTCGGGCCA AGCTGGCCAC GTGCAGCCAT AACGCCTTCC AGAAGCAGAG TTACTGCACC	60
CAGGATGTC	69
(2) INFORMATION FOR SEQ ID NO:91:	•
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 69 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:	
CCGACCTGCC TGTCTTCCCT GCTTGGCCAG CTGTCTGGCC AGGTTCGTCT GCTGCTCGGC	60
CCTCTGCAG	69
(2) INFORMATION FOR SEQ ID NO:92:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 66 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:	
CAGAGACTGC AGAGCGCCGA GCAGACGAAC CTGGCCAGAC AGCTGGCCAA GCAGGGAAGA	60
CAGGCA	60
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(2) INFORMATION FOR SEQ ID NO:93:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 70 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:	
TOTOTGOTTG GCACCCAGCT GCCGCCACAG GGCCGTACCA CTGCTCACAA GGATCCGAAC	60
GCTATCTTCC	70
(2) INFORMATION FOR SEQ ID NO:94:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 70 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:	
AAGACAGGAA GATAGCGTTC GGATCCTTGT GAGCAGTGGT ACGGCCCTGT GGCGGCAGCT	60
GGGTGCCAAG	70
(2) INFORMATION FOR SEQ ID NO:95:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 21 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:	
GGAGGAGACC AAGGCACAGG A 21	
(2) INFORMATION FOR SEQ ID NO:96:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 30 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(x1) SEQUENCE DESCRIPTION: SEQ ID NO:96:	
CCGGAATTCT TACCCTTCCT GAGACAGATT 30	
(2) INFORMATION FOR SEQ ID NO:97:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 8 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:	
CTAATGAG	8
(2) INFORMATION FOR SEQ ID NO:98:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 16 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	,
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:	
AATTCTCATT AGAGCT	16
(2) INFORMATION FOR SEQ ID NO:99:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 8 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:	
CTAATGAG .	8
(2) INFORMATION FOR SEQ ID NO:100:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 16 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:	
AATTCTCATT AGAGCT	16
(2) INFORMATION FOR SEQ ID NO:101:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 25 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:	
ATCGCCGGCT CCGCCAGCTT GTGAC	25
(2) INFORMATION FOR SEQ ID NO:102:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 30 base pairs  (B) TYPE: nucleic acid	

(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:	
GCCGAATTCT CATTAGAGCT CGTTCAGTGT	30
(2) INFORMATION FOR SEQ ID NO:103:	
<ul> <li>(i) SEQUENCE CHARACTERISTICS:</li> <li>(A) LENGTH: 42 base pairs</li> <li>(B) TYPE: nucleic acid</li> <li>(C) STRANDEDNESS: single</li> <li>(D) TOPOLOGY: linear</li> </ul>	
(ii) MÖLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:	
GATCCGAACG CTATCTTCCT GTCTTTCCAG CACCTGCTGC GT	42
(2) INFORMATION FOR SEQ ID NO:104:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 44 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:	
TTTGCCACGC AGCAGGTGCT GGAAAGACAG GAAGATAGCG TTCG	44
(2) INFORMATION FOR SEQ ID NO:105:	•
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 42 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:	
GGCAAAGTTC GTTTCCTGAT GCTGGTTGGC GGTTCTACCC TG	42
(2) INFORMATION FOR SEQ ID NO:106:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 41 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:	
ACGCACAGGG TAGAACCGCC AACCAGCATC AGGAAACGAA C	41
(2) INFORMATION FOR SEQ ID NO:107:	

(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 46 base pairs	
(B) TYPE: nucleic acid (C) STRANDEDNESS: mingle	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:	
TGCGTTCGTC GGGCGCCGCC AACCACTGCT GTTCCGTCTT AATGAA	46
(2) INFORMATION FOR SEQ ID NO:108:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 45 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:	
AGCTTTCATT AAGACGGAAC AGCAGTGGTT GGCGGCGCCC GACGA	45
(2) INFORMATION FOR SEQ ID NO:109:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 24 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:	
AAGGATCCGA ACGCTATCTT CCTG	24
(2) INFORMATION FOR SEQ ID NO:110:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 24 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:	
AGAAGCTTTC ATTAAGACGG AACA	24
(2) INFORMATION FOR SEQ ID NO:111:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 46 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:	
CTAGAAAAA CCAAGGAGGT AATAAATAAT GAAAGCACCT GTACCA	46

2) INFORMATION FOR SEQ ID NO:112:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 46 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:	
CAGGTGGTAC AGGTGCTTTC ATTATTTATT ACCTCCTTGG TTTTTT 46	
(2) INFORMATION FOR SEQ ID NO:113:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 38 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	•
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:	
CCTGCATGTG ATTTACGGGT CCTGTCTAAA CTGCTGCG 38	
(2) INFORMATION FOR SEQ ID NO:114:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 34 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:	
CGCAGCAGTT TAGACAGGAC CCGTAAATCA CATG 34	
(2) INFORMATION FOR SEQ ID NO:115:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 84 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: double  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:	
CTAGAAAAAA CCAAGGAGGT AATAAATAAT GAAAGCACCT GTACCACCTG CATGTGATTT	60
ACGGGTCCTG TCTAAACTGC TGCG	84
(2) INFORMATION FOR SEQ ID NO:116:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 20 amino acids  (B) TYPE: amino acid  (C) STRANDEDNESS:  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:
```

Pro Arg Leu Leu Asn Lys Leu Leu Arg Asp Ser Tyr Leu Leu His Arg

Arg Leu Ser Gln

- (2) INFORMATION FOR SEQ ID NO:117:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 21 amino acids
    - (B) TYPE: amino acid (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

Phe Ser Leu Gly Glu Trp Lys Thr Gln Thr Glu Gln Ser Lys Ala Gln

Asp Ile Leu Gly Ala

- (2) INFORMATION FOR SEQ ID NO:118:
  - (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 18 amino acids
    (B) TYPE: amino acid
    (C) STRANDEDNESS:

    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

Ser Arg Thr Ser Gln Leu Leu Thr Leu Asn Lys Phe Pro Asn Arg Leu

Leu Asp

- (2) INFORMATION FOR SEQ ID NO:119:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 21 amino acids (B) TYPE: amino acid

    - (C) STRANDEDNESS: (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His

Ser Arg Leu Ser Gln 20

- (2) INFORMATION FOR SEQ ID NO:120:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 16 amino acids (B) TYPE: amino acid (C) STRANDEDNESS:

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- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp 10

- (2) INFORMATION FOR SEQ ID NO: 121:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 amino acids (B) TYPE: amino acid (C) STRANDEDNESS:

    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp

Pro Asn Ala

- (2) INFORMATION FOR SEQ ID NO: 122:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 amino acids (B) TYPE: amino acid

    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr

Ala Ser Ala

- (2) INFORMATION FOR SEQ ID NO:123:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 23 amino acids
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

Ser Leu Pro Pro Asn Leu Gln Pro Gly Tyr Ser Pro Ser Pro Thr His

Pro Pro Thr Gly Gln Tyr Thr

- (2) INFORMATION FOR SEQ ID NO:124:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27 amino acids
    - (B) TYPE: amino acid (C) STRANDEDNESS:

(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: peptide	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:	
Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn T	hr Ser 15
Tyr Thr His Ser Gln Asn Leu Ser Gln Glu Gly 20 25	
(2) INFORMATION FOR SEQ ID NO:125:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 46 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:	
CTAGAAAAAA CCAAGGAGGT AATAAATAAT GAAATCTCCT GCACCA	46
(2) INFORMATION FOR SEQ ID NO:126:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 46 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:	
CAGGTGGTGC AGGAGATTTC ATTATTTATT ACCTCCTTGG TTTTTT	46
(2) INFORMATION FOR SEQ ID NO:127:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 38 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:	
CCTGCATGTG ATTTACGGGT CCTGTCTAAA CTGCTGCG	38
(2) INFORMATION FOR SEQ ID NO:128:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 34 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:	
CGCAGCAGTT TAGACAGGAC CCGTAAATCA CATG	34

(2) INFORMATION FOR SEQ ID NO:129:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 84 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: double  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:	
CTAGAAAAA CCAAGGAGGT AATAAATAAT GAAATCTCCT GCACCACCTG CATGTGA	TTT 60
ACGGGTCCTG TCTAAACTGC TGCG	84
(2) INFORMATION FOR SEQ ID NO:130:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 47 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:	
CTCCCGAACC GTACCAGCGG CCTGCTGGAA ACCAACTTTA CCGCGAG 47	
(2) INFORMATION FOR SEQ ID NO:131:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 47 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:	
GGTAAAGTTG GTTTCCAGCA GGCCGCTGGT ACGGTTCGGG AGCTCGT 47	7
(2) INFORMATION FOR SEQ ID NO:132:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 49 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:	
CGCGCGTACC ACCGGCAGCG GCCTGCTGAA ATGGCAGCAG GGCTTTCGT	49
(2) INFORMATION FOR SEQ ID NO:133:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 49 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:	
AGCCCTGCTG CCATTTCAGC AGGCCGCTGC CGGTGGTACG CGCGCTCGC	49
(2) INFORMATION FOR SEQ ID NO:134:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 50 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:	
GCGARARTCC CGGGCCTGCT GARCCAGACC AGCCGTAGCC TGGATCAGAT	50
(2) INFORMATION FOR SEQ ID NO:135:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 50 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:	
ATCCAGGCTA CGGCTGGTCT GGTTCAGCAG GCCCGGGATT TTCGCACGAA	5 <b>0</b>
(2) INFORMATION FOR SEQ ID NO:136:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 47 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:	
CCCGGGCTAT CTGAACCGTA TCCATGAACT GCTGAACGGC ACCCGTG	47
(2) INFORMATION FOR SEQ ID NO:137:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 47 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:	
GTGCCGTTCA GCAGTTCATG GATACGGTTC AGATAGCCCG GGATCTG	47
(2) INFORMATION FOR SEQ ID NO:138:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 49 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:	
GCCTGTTTCC GGGCCCGAGC CGTCGCACCC TGGGCGCGCC GGATATCAG	49
(2) INFORMATION FOR SEQ ID NO:139:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 49 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:	
ATCCGGCGCG CCCAGGGTGC GACGGCTCGG GCCCGGAAAC AGGCCACGG	49
(2) INFORMATION FOR SEQ ID NO:140:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 50 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:	
ATCAGCTCTG GCACCAGCGA TACCGGCAGC CTGCCGCCGA ACCTGCAGCC	50
(2) INFORMATION FOR SEQ ID NO:141:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 50 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:	
CAGGTTCGGC GGCAGGCTGC CGGTATCGCT GGTGCCAGAG CTGATATCCG	50
(2) INFORMATION FOR SEQ ID NO:142:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 51 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:	
GGGCTATAGC CCGAGCCCGA CCCATCCGCC GACCGGCCAG TATACCCTGT T	51
(2) INFORMATION FOR SEQ ID NO:143:	
<ul><li>(i) SEQUENCE CHARACTERISTICS:</li><li>(A) LENGTH: 51 base pairs</li><li>(B) TYPE: nucleic acid</li></ul>	

<pre>(C) STRANDEDNESS: single (D) TOPOLOGY: linear</pre>	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:	
GGTATACTGG CCGGTCGGCG GATGGGTCGG GCTCGGGCTA TAGCCCGGCT G	51
(2) INFORMATION FOR SEQ ID NO:144:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 50 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:	
TCCGCTGCCG CCGACCCCGGT GGTTCAGCTG CATCCGCTGC	50
(2) INFORMATION FOR SEQ ID NO:145:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 50 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:	
GGATGCAGCT GAACCACCGG GGTCGGCAGG GTCGGCGGCA GCGGAAACAG	50
(2) INFORMATION FOR SEQ ID NO:146:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 51 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:	
TGCCGGATCC GAGCGCGCCG ACCCCGACCC CGACCAGCCC GCTGCTGAAC A	51
(2) INFORMATION FOR SEQ ID NO:147:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 51 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:	
AGCAGCGGGC TGGTCGGGGT CGGGGTCGGC GCGCTCGGAT CCGGCAGCAG C	51
(2) INFORMATION FOR SEQ ID NO:148:	

(1) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 51 base pairs (B) TYPE: nucleic acid	
(C) STRANDEDNESS: single	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:	
CCAGCTATAC CCATAGCCAG AACCTGAGCC AGGAAGGCTA ATGAAGCTTG A	51
(2) INFORMATION FOR SEQ ID NO:149:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 51 base pairs	
(B) TYPE: nucleic acid (C) STRANDEDNESS: single	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:	
CTTCATTAGC CTTCCTGGCT CAGGTTCTGG CTATGGGTAT AGCTGGTGTT C	51
(2) INFORMATION FOR SEQ ID NO:150:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 21 base pairs	
(B) TYPE: nucleic acid	
(C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(0),101020011 25.1022	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:	
ACGAGCTCCC GAACCGTACC A	21
(2) INFORMATION FOR SEQ ID NO:151:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 21 base pairs	
(B) TYPE: nucleic acid	
(C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(5)	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:	
CTGATATCCG GCGCGCCCAG G	21
(2) INFORMATION FOR SEQ ID NO:152:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 base pairs	
(B) TYPE: nucleic acid	
(C) STRANDEDNESS: single	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:	
CGGATATCAG CTCTGGCACC A	21

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(2) INFORMATION FOR SEQ ID NO:153:

	(1)	) SE(	(A) (B) (C)	LEN TYI STI	NGTH: PE: 1 RANDI	21 Duclo EDNES	Dase base SS: line	pa: acid								
		L) MO														
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:153: CARGCTTCA TTAGCCTTCC T															
(CA)	AGCT:	CA 7	TAG	CTT	CC T							•			21	
(2)	INF	ORMA	NOIT	FOR	SEQ	ID I	NO: 1	54:								
	(i)	) SE(	(A (B (C	) LEI ) TYI ) STI	NGTH PE: 1 RANDI	: 490 nucle EDNE:	ISTIC 0 ba: 8ic : SS: c line:	se pa acid doub								
	(ii) MOLECULE TYPE: Other nucleic acid (xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:															
	( <b>x</b> :	i) SI	EQUE	NCE I	ESCI	RIPT	ION:	SEQ	ID I	NO: 15	54:					
CTC Leu 1	CCG Pro	AAC Asn	CGT Arg	ACC Thr 5	AGC Ser	GGC Gly	CTG Leu	CTG Leu	GAA Glu 10	ACC Thr	AAC Asn	TTT Phe	ACC Thr	GCG Ala 15	AGC Ser	48
CG 11a	CGT Arg	ACC Thr	ACC Thr 20	GCC GLY	AGC Ser	GGC Gly	CTG Leu	CTG Leu 25	AAA Lys	TGG Trp	CAG Gln	CAG Gln	30 GLy GGC	TTT Phe	CGT Arg	96
77 a	AAA Lys	ATC Ile 35	CCG Pro	GGC Gly	CTG Leu	CTG Leu	AAC Asn 40	CAG Gln	ACC Thr	AGC Ser	CGT Arg	AGC Ser 45	CTG Leu	GAT Asp	CAG Gln	144
ATC [le	CCG Pro 50	GGC	TAT Tyr	CTG Leu	AAC Asn	CGT Arg 55	ATC Ile	CAT His	GAA Glu	CTG Leu	CTG Leu 60	AAC Asn	GCC	ACC Thr	CGT Arg	192
GC Gly 65	CTG Leu	TTT Phe	CCG Pro	GGC Gly	CCG Pro 70	AGC	CGT Arg	CGC Arg	ACC Thr	CTG Leu 75	GGC Gly	GCG Ala	CCG Pro	GAT Asp	ATC Ile 80	240
AGC Ser	TCT Ser	GGC	ACC Thr	AGC Ser 85	GAT Asp	ACC Thr	GJ Y	AGC Ser	CTG Leu 90	CCG Pro	CCG Pro	AAC Asn	CTG Leu	CAG Gln 95	CCG Pro	288
GC Sly	TAT Tyr	AGC Ser	CCG Pro 100	AGC Ser	CCG Pro	ACC Thr	CAT His	CCG Pro 105	CCG Pro	ACC Thr	GGC Gly	CAG Gln	TAT Tyr 110	ACC Thr	CTG Leu	336
rrr ?he	PIO	CTG Leu 115	CCG Pro	CCG Pro	ACC Thr	CTG Leu	CCG Pro 120	ACC Thr	CCG Pro	GTG Val	GTT Val	CAG Gln 125	CTG Leu	CAT His	CCG Pro	384
CTG Leu	CTG Leu 130	CCG Pro	GAT Asp	CCG Pro	AGC Ser	GCG Ala 135	CCG Pro	ACC Thr	CCG Pro	ACC Thr	CCG Pro 140	ACC Thr	AGC Ser	CCG Pro	CTG Leu	432
CTG Leu 145	AAC Asn	ACC Thr	AGC Ser	TAT. Tyr	ACC Thr 145	CAT His	AGC Ser	CAG Gln	AAC Asn	CTG Leu 150	AGC Ser	CAG Gln	GAA Glu	GGC G1y	TAA	480
IGA	AGCT	IGA														490

(2) INFORMATION FOR SEQ ID NO:155:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 24 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:	
AAGGATCCGA ACGCTATCTT CCTG	24
(2) INFORMATION FOR SEQ ID NO:156:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 56 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:	
GGGAGCTCGT TCAGGGTCAG AACCAGAGAG GTACGAGACG GAACAGCAGT GG	TTGG 56
(2) INFORMATION FOR SEQ ID NO:157:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 157 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: double  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:	
G GAT CCG AAC GCT ATC TTC CTG TCT TTC CAG CAC CTG CTG CGT Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg 1 5 10	GGC 46 Gly 15
AAA GTT CGT TTC CTG ATG CTG GTT GGC GGT TCT ACC CTG TGC G Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys V 20 . 25	TT CGT 94 al Arg 30
CGG GCG CCG ACC ACT GCT GTT CCG TCT CGT ACC TCT CTG G Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu V 35 40 45	TT CTG 142 al Leu
ACC CTG AAC GAG CTC Thr Leu Asn Glu Leu 50	157
(2) INFORMATION FOR SEQ ID NO:158:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 100 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	

(ii) MOLECULE TYPE: Other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:	
CCTCGAGGAA TTCCTGCAGC CCGGGACTAG TATCGGCTAC CCCTACGACG TCCCCGACTA	6
CGCCGGCGTC CATCACCATC ACCATCACTG AGCGGCCGCC	10
(2) INFORMATION FOR SEQ ID NO:159:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 108 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) HOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:	
AATTGGCGGC CGCTCAGTGA TGGTGATGGT GATGAGCGCC GGCGTAGTCG GGGACGTCGT	6
AGGGGTAGCC GATACTAGTC CCGGGCTGCA GGAATTCCTC GAGGGTAC	10
(2) INFORMATION FOR SEQ ID NO:160:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 70 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:160:	
AATTCCAAGA TCTCACACTT GCTTTTGACA CAACTGTGTT TACTTGCAAT CCCCCAAAAC	60
AGACAGACCC	70
(2) INFORMATION FOR SEQ ID NO:161:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 66 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:161:	
GGGTCTGTCT GTTTTGGGGG ATTGCAAGTA AACACAGTTG TGTCAAAAGC AAGTGTGAGA	60
TCTTGG	66
(2) INFORMATION FOR SEQ ID NO:162:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 29 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:162:	
GGCCCGGGAT GGAGCTGACT GAATTCCTC	

(2) INFORMATION FOR SEQ ID NO:163:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 35 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:163:	
TCAAGCTTAC TAGTCCCTTC CTGAGACAGA TTCTG	35
(2) INFORMATION FOR SEQ ID NO:164:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 22 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:164:	
TARTACGACT CACTATAGGG CG	22
(2) INFORMATION FOR SEQ ID NO:165:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 22 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:165:	
AATTCCAAGA TCACACACTT GC	22
(2) INFORMATION FOR SEQ ID NO:166:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 35 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:166:	
TCAAGCTTAC TAGTCCCTTC CTGAGACAGA TTCTG	35
(2) INFORMATION FOR SEQ ID NO:167:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 35 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single	

(ii) MOLECULE TYPE: Other nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:167:

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TGGGCACTGG CTCAGGTTGC TGTGAAGGAC ATGGG 35 (2) INFORMATION FOR SEQ ID NO:168: (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: Other nucleic acid (xi) SEQUENCE DESCRIPTION: SEQ ID NO:168: TGTAGGCAAA GGGGTAACCT CTGGGCA 27 (2) INFORMATION FOR SEQ ID NO:169: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:169: Thr Ser Ile Gly Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Gly Val His 10 Xaa Xaa Xaa Xaa Xaa 20 . (2) INFORMATION FOR SEQ ID NO:170: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 30 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: Other nucleic acid (xi) SEQUENCE DESCRIPTION: SEQ ID NO:170: TTTGAATTCG GCCAGCCAGA CACCCCGGCC 30 (2) INFORMATION FOR SEQ ID NO:171: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 41 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: Other nucleic acid (xi) SEQUENCE DESCRIPTION: SEQ ID NO:171: TTTGCGGCCG CTCATTATTC GTGTATCCTG TTCAGGTATC C 41 (2) INFORMATION FOR SEQ ID NO:172: (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs (B) TYPE: nucleic acid

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(C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:172:	
TGTAGGCAAA GGAACCTCTG GGCA	24
(2) INFORMATION FOR SEQ ID NO:173:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 27 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:173:	
TGTAGGCAÄA GGAGTGTGAA CCTCTGG	27
(2) INFORMATION FOR SEQ ID NO:174:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 27 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:174:	
TGTAGGCAAA GGAGCGTGAA CCTCTGG	27
(2) INFORMATION FOR SEQ ID NO:175:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 27 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:175:	
TGTAGGCAAA GGTCCGTGAA CCTCTGG	27
(2) INFORMATION FOR SEQ ID NO:176:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 24 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:176:	
AGCTGTGGTC CTCTGTGGAG GAAG	. 24
(2) INFORMATION FOR SEQ ID NO:177:	

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(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 24 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:177:	
GTGAGCTGTG GTGCCCTGTG GAGG	24
(2) INFORMATION FOR SEQ ID NO:178:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 27 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:178:	
AGCTGTGGTC CTGTTGCCCT GTGGAGG	27
(2) INFORMATION FOR SEQ ID NO:179:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 27 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:179:	
AGCTGTGGTC CTAGCGCCCT GTGGAGG	27
(2) INFORMATION FOR SEQ ID NO:180:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 27 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:180:	
AGCTGTGGTC CTTCCGCCCT GTGGAGG	27
(2) INFORMATION FOR SEQ ID NO:181:	
(i) SEQUENCE CHARACTERISTICS:  (A) LENGTH: 26 base pairs  (B) TYPE: nucleic acid  (C) STRANDEDNESS: single  (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: Other nucleic acid	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:181:	
CGGGCTGCAG GATATCCAAG ATCTCA	26

(ix) FEATURE:

(A) NAME/KEY: misc feature (D) OTHER INFORMATION:

```
(2) INFORMATION FOR SEQ ID NO:182:
       (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
                 (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE: Other nucleic acid
       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:182:
TAATACGACT CACTATAGGG CG
                                                                         22
(2) INFORMATION FOR SEQ ID NO:183:
       (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 34 base pairs (B) TYPE: nucleic acid
                (C) STRANDEDNESS: Bingle
                (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE: Other nucleic acid
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:183:
GGGCGGCCGC TCAGCTGGGG ACAGCTGTGG TGGG
                                                                        34
(2) INFORMATION FOR SEQ ID NO:184:
      (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 7 amino acids
                (B) TYPE: amino acid
(C) STRANDEDNESS:
                (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE: peptide
     (ix) FEATURE:
            (A) NAME/KEY: misc feature (D) OTHER INFORMATION:
                   /notes "The amino acid at position 2 is
Ala, Ser, Gly, Met or Gln."
     (ix) FEATURE:
            (A) NAME/KEY: misc feature (D) OTHER INFORMATION:
                    /note= "The amino acid at position 7 is
                             Glu or Lys."
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:184:
Lys Xaa Tyr Tyr Glu Ser Xaa
(2) INFORMATION FOR SEQ ID NO:185:
      (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 6 amino acids
(B) TYPE: amino acid
               (C) STRANDEDNESS:
(D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: peptide
```

/note= "The amino acid at position 6 is Glu or Asp."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:185:

Lys Glx Arg Ala Ala Xaa

- (2) INFORMATION FOR SEQ ID NO:186: .
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 7 amino acids
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
    - (ii) MOLECULE TYPE: peptide
    - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:186:

Lys Ala Gly Xaa Cys Ser Gly

- (2) INFORMATION FOR SEQ ID NO:187:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 13 amino acids
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (ix) FEATURE:
    - (A) NAME/KEY: misc\_feature (D) OTHER INFORMATION:

/note= "The amino acid at position 2 is Ile, Thr or Ser."

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:187:
- Lys Xaa Pro Val Pro Pro Ala Cys Asp Pro Arg Leu Leu
- (2) INFORMATION FOR SEQ ID NO:188:
  - (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 9 amino acids

    - (B) TYPE: amino acid

    - (C) STRANDEDNESS: (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:188:

Lys Asp Ser Phe Leu Ala Asp Val Lys

- (2) INFORMATION FOR SEQ ID NO:189:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 9 amino acids
      (B) TYPE: amino acid

    - (C) STRANDEDNESS:

- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (ix) FEATURE:
  - (A) NAME/KEY: misc feature
  - (D) OTHER INFORMATION:

/note= "The amino acid at position 1 is Lysine or Arginine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:189:

Xaa Thr Leu Pro Thr Xaa Ala Val Pro

- (2) INFORMATION FOR SEQ ID NO:190:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 15 amino acids (B) TYPE: amino acid

    - (C) STRANDEDNESS: (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:190:

Lys Asp Ser Phe Leu Ala Asp Val Lys Gln Tyr Tyr Glu Ser Glu

- (2) INFORMATION FOR SEQ ID NO:191:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 332 amino acids (B) TYPE: amino acid

    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (vi) ORIGINAL SOURCE:
    - (A) ORGANISM: Homo Sapiens
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:191:

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu 1 5 10

Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val 20 25 30

His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu

Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu

Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln 65 70 75 80

Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln

Val Arg Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu

Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe

											140				Leu	
										473					Ala 160	
											Asn			175		
											Ala		190			
Thr	Gly	Ser 195	Gly	Leu	Leu	Lye	Trp 200	Gln	Gln	Gly	Phe	Airg 205	Ala	Lys	Ile	
											Asp 220				_	
Tyr 225	Leu	Asn	Arg	Ile	His 230	Glu	Leu	Leu	Asn	Gly 235	Thr	Arg	Gly	Leu	Phe 240	
Pro	Gly	Pro	Ser	Arg 245	Arg	Thr	Leu	Gly	Ala 250	Pro	Asp	Ile	Ser	Ser 255	Gly	
											Gln		270			
												285				
											His 300					
										313	Pro :	Leu	Leu .		Thr 320	
Ser	Tyr	Thr	His :	Ser 325	Gln i	Asn :	Leu	Ser	Gln 330	Glu ·	Gly					
(2)	Info	RMAT	ION :	FOR	SEQ :	ID N	0:19	2:								
	(i)	SEQ	(A) (B) (C)	TYP:	ARAC: GTH: E: ni ANDEI OLOGI	1080 electrones	6 ba: ic a: S: d:	se p. cid								•
	(ii	) MOI	LECUI	E T	YPE:	CDN	A to	mRN	A							
	(vi	) OR:	IGINA (A)	L SO	Durce Anish	E: 4: Ho	omo s	Sapi	ens							
	(xi	SEC	QUENC	E DI	ESCRI	PTIC	on: s	SEQ :	D NO	: 192	2:					
GGCC	AGCC	AG AG	CACCO	CGG	CAG	A AT Me -2	TG GA St G1 21 -2	NG CT	rg ac	T G	AA TI Lu Le	u Le -1	u Le 5	u Va	.1	51
GTC 1	-	-10					-5			.eu S	er S	er p l	ro A	la P	ro	99
Pro F					10				ys L	15	eu A	rg A	sp s	er H	18 20	147
GTC (	TT C	AC A	GC A	GA C	TG A	ec c	AG T	GC C	CA G	AG G	TT C	AC C	CT T	TG C	CT	195

Val	Leu	Hie	Ser	Arg 25	Leu	Ser	Gln	Cys	Pro 30	Glu	Val	His	Pro	Leu 35	Pro	
ACA Thr	Pro	GTC Val	CTG Leu 40	CTG Leu	CCT Pro	GCT	GTG Val	GAC Asp 45	TTT Phe	AGC Ser	TIG	GGA Gly	GAA Glu 50	Trp	AAA Lys	243
ACC Thr	CAG Gln	ATO Met		GAG Glu	ACC Thr	AAG Lys	GCA Ala 60	GIN	GAC Asp	ATT	CTG	GGA Gly 65	Ala	GTG Val	ACC Thr	291
	70	-	. 010	GIY	VAI	75	ATE	ALE	Arg	GIA	61n 80	Leu	Gly	Pro	ACT Thr	339
85		561	261	240	90	GIY	GIR	Lea	ser	95 95	Gln	Val	Arg	Leu	CTC Leu 100	387
	J.,	n L a	Leu	105	Ser	Leu	Leu	GIÀ	110	Gln	Leu	Pro	Pro	Gln 115	GGC Gly	435
9	••••		120	ure	Lys	GAT Amp	PTO	125	Ala	Ile	Phe	Leu	Ser 130	Phe	Gln	483
		135	nry	Uly	Lys	GTG Val	140	Pne	Leu	Met	Leu	Val 145	Gly	Gly	Ser	531
	150	Cys	461	ira	Arg	GCC Ala 155	Pro	Pro	Thr	Thr	Ala 160	Val	Pro	Ser	Arg	579
165	<del>-</del>	200	,41	260	170	CTG Leu	ASU	GIU	Leu	Pro 175	Asn	Arg	Thr	Ser	Gly 180	627
		010	1111	185	Pne	ACT Thr	ATA	Ser	190	Arg	Thr	Thr	Gly	Ser 195	Gly	675
		2,5	200	GIII	GIN	GGA Gly	Pne	Arg 205	Ala	Lys	Ile	Pro	Gly 210	Leu	Leu	723
		215	Jer	AL Y	361		220	GIN	Ile	Pro	Gly	Tyr 225	Leu	Asn	Arg	771
	230	<b>U</b> 10	Ded	Leu	nan	GGA Gly 235	inr	Arg	GIÀ	Leu	Phe 240	Pro	GlŸ	Pro	Ser	819
245	· y			u.,	250	CCG Pro	veb	116	ser	Ser 255	Gly	Thr	Ser	yab	Thr 260	867
•		,-		265	A-11	CTC Leu	<b>U</b> 111	Pro	270	Tyr	Ser	Pro	Ser	Pro 275	Thr	915
CAT His	CCT Pro	CCT Pro	ACT Thr 280	GGA Gly	CAG Gln	TAT Tyr	Inr .	CTC Leu 285	TTC Phe	CCT Pro	CTT Leu	Pro	CCC Pro 290	ACC Thr	TTG Leu	963

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Pro	ACC Thr	CCT Pro 295	GTG Val	GTC Val	CAG Gln	CTC Leu	CAC His 300	CCC Pro	CTG Leu	CTT Leu	CCT Pro	GAC Asp 305	CCT Pro	TCT Ser	GCT Ala		1011
										AAC Asn							1059
						GAA Glu		TAA									1086
(2)	INFO	RMAI	MOI	FOR	SEQ	ID N	10:19	3:				•					
	(i)	SE	(A) (B) (C)	LEN TYP STR	GTH: PE: 6 VANDI	TERI 7 a mino DNES GY: 1	mino aci S: s	ac: id sing:									
	(i.i	.) M	DLEC	JLE 1	TYPE:	: pe	ptide	•									
	(×:	.) SI	EQUE	NCE I	ESC	RIPT	ON:	SEQ	ID I	NO:19	93:						
Lys 1	Gln	Tyr	Tyr	Glu S	Ser	Glu											
(2)	INF	ORMA!	TION	FOR	SEQ	ID !	10:19	94:									
	(i)	SE(	(A (B (C	) LEI ) TYI ) STI	NGTH PE: : RAND:	CTER: : 160 nuclo EDNE: GY:	63 ba eic a SS: 0	ase acid doub		s							
	(i.	i) M	OLEC	ULE '	IYPE	: cD	NA t	o mR	NA								
	(v.	i) O	(A		GANI					gicu	5						
	( x	i) 5	EQUE	NCE	DESC	RIPT	ION:	SEQ	ID	NO:1	94:						
GGT	GTAC	CTG	GGTC	CTGA	AG C	CCTT	CTTC	A CC	TGGA	TAGA	TTC	CTTG	GCC	CACC	TGTC	cc	60
CAC	CCCA	CTC	TGTG	CAGA	GG I	ACAA	AAGC	T CA	AGCC	GTCT	CCA	TGGC	ccc	AGGA	AAGA:	TT	120
CAG	GGGA	GAG	GCCC	CACA	CA G	GGAG	CCAC	T GC	AGTO	AGAC	ACC	CTGG	GCA	M	TG et 21		175
	Leu					Leu					Lev				AGA Arg -5		223
					Pro			Pro						Lev	CTA Leu		271
			Lev					Lev					Le		CAG Gln		319
		Ası					ı Sez					ı Le			r GTG a Val		36

GAC 1 Asp 1 45	rne .	ser	reu	GIY	50	Trp	Lys	Thr	Gln	Thr 55	Glu	Gln	Ser	Lys	Ala 60	419
CAG ( Gln )	GAC . Asp	ATT Ile	CTA Leu	GGG Gly 65	GCA Ala	GTG Val	TCC Ser	CTT Leu	CTA Leu 70	CTG Leu	GAG Glu	GGG Gly	GTG Val	ATG Met 75	GCA Ala	463
GCA ( Ala i	CGA (	GGA Gly	CAG Gln 80	TTG Leu	GAA Glu	CCC Pro	TCC Ser	TGC Cys 85	CTC Leu	TCA Ser	TCC Ser	CTC Leu	CTG Leu 90	Gly	CAG Gln	511
CTT : Leu :	TCT ( Ser (	GGT Gly 95	CAG Gln	GTT Val	CGC Arg	CTC Leu	CTC Leu 100	TTG Leu	GGA Gly	GCC Ala	CTG Leu	CAG Gln 105	GGC	CTC Leu	CTA Leu	555
GGA 1 Gly 1	ACC ( Thr (	CAG Gln	GTA Val	AGT Ser	CCC Pro	CAG Gln 115	ACC Thr	TAT Tyr	AGA Arg	AAC Asn	TAC Tyr 120	CCT Pro	CTT Leu	ACT Thr	CAG Gln	607
TTC ( Phe 1 125	CTC Leu	TAAC	GAC	TG C	GAAJ	<b>LAGA</b> (	A AC	GGAT	TCT	GA1	TCT	(GGT	GTC	TTCA	TG:	663
TATG	AAAG	CT C	GTC1	CATAC	G G	AGTG	TGC	TCI	CAGO	CAC	AATA	CCTC	GG '	IGCT	GCAG	723
TAAA	CTTT	CC P	CCTI	AGTO	A G	AGAC	GCC1	CA2	TATGT	rece	CCA	CTC	CT (	GCC:	CAGG	783
CCAT	CCTC	TG C	CTTC	AGCT	T C	CTCCA	CAGO	GC)	AGGAC	CAC	AGCT	CAC	AAG (	GACC	CAGTO	843
CCCT	CTTC	TT G	AGCI	TGC	LA CA	LACTO	CTT	GGG	GAAA	GGT	GCGC	TTC	TG (	CTGC	GGTAC	903
AAGG:	TCCC	GC C	CTCI	GTG1	C AC	ACGG	ACC	TIC	CCAC	CAC	AGC1	CTC	CA	AGCAG	AACCI	963
CTCA	ACTC	CT C	ACAC	TAAF	LC AJ	GTT	CCAP	ACA	GACT	TCT	GGAT	TGT	rec i	AGACO	AACTI	1023
CAGT	GTTG:	TA G	CCAG	AACI	'G C1	GGCC	CTGG	ACT	TCTG	AAC	AGGC	TTC	AG (	GATTO	CAGAGO	1083
CAAG	ATTA:	IT C	CTGG	TCAC	C TA	CTAAL	AAAC	CTC	cccc	TCC	TTAC	ACCA	AA :	rccc	GGATA	1143
CCTG	AACG	GG A	CACA	CGA	vc C1	GTGA	ATG	AAC	TCAT	GGG	CTCI	TTGC	CTG (	GAC	TCACI	1203
ACAGI	ACCC:	TG G	AAGC	:CCC#	G AC	GTTC	TGCC	AG0	AGCI	TTC	AACA	AAGO	CT (	CTTC	CCACI	1263
CAAC	CTCC	AG A	GTGG	ACTI	C C1	CCTA	TCC	AA C	CCTI	CCT	GCT	ATGO	AT I	ACAC	CTTTI	1323
CCCT	CCTT	CA C	CTAC	CTTC	c co	ACCO	CTGG	GIC	TCC	ccc	CAGO	TCCC	cc (	CCGT	TCCT	; 1383
ACCC	CTCC	AC C	ACCA	TACC	T A	CTCI	ACC	ACC	CTC	TCC	AGG	CTTC	GT (	CTCAC	TAAGO	1443
GTCC	CGTG	CA C	TGG	ACGO	A GO	GCGF	TCGI	CTC	CAAC	ATC	TCT	AGGC	GC I	AAGĊ	TCCT	1503
AGGAJ	AGGC'	TC 1	GAGO	CAGO	T C	CTAC	ACAT	. cc	CCTC	TCG	CCT	ACGO	GC (	CCTG	GAAAG	1563
GGAT	ACAC	AG G	CCAC	GAC	C TO	TAC	ACC1	TAC	GAGO	GAT	TTTI	TTCI	MTA I	ACCT	TCAAC	1623
AATA:	TTCA'	TC A	GAGC	AAA:	A AJ	<b>LAAA</b>	LAAA.	AAA A	AAA.	AAA			•			1663

## (2) INFORMATION FOR SEQ ID NO:195:

- (i) SEQUENCE CHARACTERISTICS:

  (A) LENGTH: 861 base pairs

  (B) TYPE: nucleic acid

  (C) STRANDEDNESS: double

  (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo Sapiens
 (F) TISSUE TYPE: liver

# (xi) SEQUENCE DESCRIPTION: SEQ ID NO:195:

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GTC ATG CTT CTC C Val Met Leu Leu I -10	CTA ACT GCA AGG Leu Thr Ala Arg	Leu Thr Leu	TCC AGC CCG GCT Ser Ser Pro Ala	CCT 99
CCT GCT TGT GAC C Pro Ala Cys Asp I 5	CTC CGA GTC CTC Leu Arg Val Leu 10	AGT AAA CTG Ser Lys Leu 15	CTT CGT GAC TCC Leu Arg Asp Ser	CAT 147 His 20
GTC CTT CAC AGC A	AGA CTG AGC CAG Arg Leu Ser Gln 25	TGC CCA GAG Cys Pro Glu 30	GTT CAC CCT TTG Val His Pro Leu 35	CCT 195 Pro
ACA CCT GTC CTG C Thr Pro Val Leu I 40	CTG CCT GCT GTG Leu Pro Ala Val	GAC TIT AGC Asp Phe Ser 45	TTG GGA GAA TGG Leu Gly Glu Trp 50	AAA 243 Lys
ACC CAG ATG GAG G Thr Gln Met Glu G 55	AG ACC AAG GCA Slu Thr Lys Ala 60	Gln Asp Ile	CTG GGA GCA GTG Leu Gly Ala Val 65	ACC 291 Thr
CTT CTG CTG GAG C Leu Leu Leu Glu C 70	GGA GTG ATG GCA Gly Val Met Ala 75	GCA CGG GGA Ala Arg Gly	CAA CTG GGA CCC Gln Leu Gly Pro 80	ACT 339 Thr
TGC CTC TCA TCC C Cys Leu Ser Ser I 85	CTC CTG GGG CAC Leu Leu Gly Glr 90	CTT TCT GGA Leu Ser Gly 95	CAG GTC CGT CTC Gln Val Arg Leu	CTC 387 Leu 100
CTT GGG GCC CTG ( Leu Gly Ala Leu (	CAG AGC CTC CT1 Sin Ser Leu Leu 105	GGA ACC CAG Gly Thr Gln 110	CTT CCT CCA CAG Leu Pro Pro Gln 115	GGC 435 Gly
AGG ACC ACA GCT ( Arg Thr Thr Ala I 120	CAC AAG GAT CCC His Lys Asp Pro	AAT GCC ATC Asn Ala Ile 125	TTC CTG AGC TTC Phe Leu Ser Phe 130	CAA 483 Gln
CAC CTG CTC CGA ( His Leu Leu Arg ( 135	GGA AAG GTG CGT Gly Lys Val Arc 140	J Phe Leu Met	CTT GTA GGA GGG Leu Val Gly Gly 145	TCC 531 Ser
ACC CTC TGC GTC : Thr Leu Cys Val : 150	AGG CGG GCC CCA Arg Arg Ala Pro 155	A CCC ACC ACA Pro Thr Thr	GCT GTC CCC AGC Ala Val Pro Ser 160	AGA 579 Arg
ACC TCT CTA GTC ( Thr Ser Leu Val ) 165	CTC ACA CTG AAG Leu Thr Leu Ass 170	GAG CTC CCA Glu Leu Pro 175	Asn Arg Thr Ser	GGA 627 Gly 180
TTG TTG GAG ACA : Leu Leu Glu Thr :	AAC TTC ACT GC: Asn Phe Thr Al: 185	TCA GCC AGA a Ser Ala Arg 190	ACT ACT GGC TCT Thr Thr Gly Ser 195	GGG 675
CTT CTG AAG TGG Leu Leu Lys Trp	Gln Gln Gly Ph	C AGA GCC AAG 8 Arg Ala Lys 205	ATT CCT GGT CTG Ile Pro Gly Leu 210	CTG 723 Leu
AAC CAA ACC TCC	AGG TCC CTG GA	C CAA ATC CCC	GGA TAC CTG AAC	AGG 771

Asn	Gln	Thr 215	Ser	Arg	Ser	Leu	Asp 220	Gln	Ile	Pro	Gly	Tyr 225	Leu	Asn	Arg	
ATA Ile	CAC His 230	GAA Glu	CTC Leu	TTA	LAAA	AAA ;	AAAA	AAAA	AA A	AAAA	AAAA	A AA.	AAAA	AAAA		823
AAA	AAAA	AAA I	AAAA	AAAA	LA AJ	AAA	AAAA	A AA	AAAA	<b>A</b> A						861
(2)	INF	ORMA:	rion	FOR	SEQ	ID i	NO:1	96:								
	(i)	) SE	(A (B (C	) LEI ) TYI ) STI	NGTH: PE: : RAND!	12 nucl DNE	ISTIC 67 ba eic a SS: c linea	ase ; acid doub.	_	s						
	(i:	i) M	DLEC	ULE 1	TYPE:	CD:	NA to	o muRi	NA							
	(V:	i) O	(A	NAL S ORG TIS	CANIS	SM: 1	Homo E: 1:	Sap: iver	iens							
	(×:	i) Si	EQUE	NCE I	DESC	RIPT	ION:	SEQ	ID I	NO: 19	96:					•
GGC	CAGC	CAG I	ACAC	cccc	sc cı	1	ATG ( Met ( -21 -	Glu 1	CTG 1	ACT (	GAA : Glu I	Leu 1	CTC ( Leu ) -15	CTC ( Leu '	GTG Val	51
GTC Val	ATG Met	CTT Leu -10	CTC Leu	CTA Leu	ACT Thr	GCA Ala	AGG Arg -5	CTA Leu	ACG Thr	CTG Leu	TCC Ser	AGC Ser 1	CCG Pro	GCT Ala	CCT Pro	99
CCT Pro 5	GCT Ala	TGT Cys	GAC Asp	·CTC Leu	CGA Arg 10	GTC Val	CTC Leu	AGT Ser	AAA Lys	CTG Leu 15	CTT Leu	CGT Arg	GAC Asp	TCC Ser	CAT His 20	147
GTC Val	CTT Leu	CAC His	AGC Ser	AGA Arg 25	CTG Leu	AGC Ser	CAG Gln	TGC Cys	CCA Pro 30	GAG Glu	GTT Val	CAC His	CCT Pro	TTG Leu 35	CCT Pro	195
ACA Thr	CCT Pro	GTC Val	CTG Leu 40	CTG Leu	CCT Pro	GCT Ala	GTG Val	GAC Asp 45	TTT	AGC Ser	TTG Leu	GGA Gly	GAA Glu 50	TGG Trp	AAA Lys	243
ACC Thr	CAG Gln	ATG Met 55	GAG Glu	GAG Glu	ACC Thr	AAG Lys	GCA Ala 60	CAG Gln	GAC Asp	ATT Ile	CTG Leu	GGA Gly 65	GCA Ala	GTG Val	ACC Thr	291
CTT Leu	CTG Leu 70	CTG Leu	GAG Glu	GGA Gly	GTG Val	ATG Met 75	GCA Ala	GCA Ala	CGG Arg	GGA Gly	CAA Gln 80	CTG Leu	GGA Gly	CCC Pro	ACT Thr	339
TGC Cys 85	CTC Leu	TCA Ser	Ser	CTC Leu	Leu	Gly	CAG Gln	Leu	Ser	GGA Gly 95	Gln	GTC Val	CGT Arg	CTC Leu	CTC Leu 100	387
CTT Leu	GGG Gly	GCC Ala	CTG Leu	CAG Gln 105	AGC Ser	CTC Leu	CTT Leu	GGA Gly	ACC Thr 110	CAG Gln	CTT Leu	CCT Pro	CCA Pro	CAG Gln 115	GGC Gly	435
AGG Arg	ACC Thr	ACA Thr	GCT Ala 120	CAC His	AAG Lys	GAT Asp	CCC Pro	AAT Asn 125	GCC Ala	ATC Ile	TTC Phe	CTG Leu	AGC Ser 130	TTC Phe	CAA Gln	483
CAC His	CTG Leu	CTC Leu	CGA Arg	GGA Gly	AAG Lys	GTG Val	CGT Arg	TTC Phe	CTG Leu	ATG Met	CTT Leu	GTA Val	GGA Gly	GGG Gly	TCC Ser	531

		135	•				140		•			145					
ACC Thr	CTC Leu 150	TGC Cys	GTC Val	AGG Arg	CGG Arg	GCC Ala 155	CCA Pro	CCC Pro	ACC Thr	ACA Thr	GCT Ala 160	GTC Val	CCC Pro	AGC Ser	AGA Arg		579
ACC Thr 165	TCT Ser	CTA Leu	GTC Val	CTC Leu	ACA Thr 170	CTG Leu	AAC Asn	GAG Glu	CTC Leu	CCA Pro 175	AAC Asn	AGG Arg	ACT Thr	TCT Ser	GGA Gly 180	ı	627
TTG Leu	TTG Leu	GAG Glu	ACA Thr	AAC Asn 185	TTC Phe	ACT Thr	GCC Ala	TCA Ser	GCC Ala 190	AGA Arg	ACT Thr	ACT Thr	GGC Gly	TCT Ser 195	GGG Gly	I	675
CTT Leu	CTG Leu	AAG Lys	TGG Trp 200	CAG Gln	CAG Gln	GGA Gly	TTC Phe	AGA Arg 205	GCC Ala	AAG Lys	ATT Ile	CCT Pro	GGT Gly 210	CTG Leu	CTG Leu	•	723
AAC Asn	CAA Gln	ACC Thr 215	TCC Ser	AGG Arg	TCC Ser	CTG Leu	GAC Asp 220	CAA Gln	ATC Ile	CCC Pro	GGA Gly	TAC Tyr 225	CTG Leu	AAC Asn	AGG Arg		771
ATA Ile	CAC His 230	GAA Glu	CTC Leu	TTG Leu	AAT Asn	GGA Gly 235	ACT Thr	CGT Arg	GGA Gly	CTC Leu	TTT Phe 240	CCT Pro	GGA Gly	CCC Pro	TCA Ser	;	819
CGC Arg 245	AGG Arg	ACC Thr	CTA Leu	GGA Gly	GCC Ala 250	CCG Pro	GAC Asp	ATT	TCC Ser	TCA Ser 255	GGA Gly	ACA Thr	TCA Ser	GAC Asp	ACA Thr 260	Į	867
GCC	TCC Ser	CTG Leu	CCA Pro	CCC Pro 265	AAC Asn	CTC Leu	CAG Gln	CCT Pro	GGA Gly 270	TAT Tyr	TCT Ser	CCT Pro	TCC Ser	CCA Pro 275	ACC Thr	•	915
CAT His	CCT Pro	CCT Pro	ACT Thr 280	GGA Gly	CAG Gln	TAT Tyr	ACG Thr	CTC Leu 285	TTC Phe	CCT Pro	CTT Leu	CCA Pro	CCC Pro 290	ACC Thr	TTG Leu	•	963
CCC Pro	ACC Thr	CCT Pro 295	GTG Val	GTC Val	CAG Gln	CTC Leu	CAC His 300	CCC Pro	CTG Leu	CTT Leu	CCT Pro	GAC Asp 305	CCT Pro	TCT Ser	GCT Ala	10	011
CCA Pro	ACG Thr 310	CCC Pro	ACC Thr	CCT Pro	ACC Thr	AGC Ser 315	CCT Pro	CTT Leu	CTA Leu	AAC Asn	ACA Thr 320	TCC Ser	TAC Tyr	ACC Thr	CAC His	10	059
TCC Ser 325	CAG Gln	AAT Asn	CTG Leu	TCT Ser	CAG Gln 330	GAA Glu	GCG	TAAC	GTT	CTC A	(GAC	CTG	C G	CATO	AGCA	1:	113
TTG	CTC	ATG 1	CACAC	CTC	C TI	rccc	rgcad	GGG	ccc	CTG	GGAG	JACAI	ACT (	GAC	LAGAT:	r 1:	173
TCCTACTTC TCCTGAAACC CAAAGCCCTG GTAAAAGGGA TACACAGGAC TGAAAAGGGA 1									233								
ATCATTTTTC ACTGTACATT ATAAACCTTC AGAA 1									267								

## (2) INFORMATION FOR SEQ ID NO:197:

- (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 549 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: synthetic DNA

(XŢ)	SEQUENCE	DESCRIPTION:	SEQ	ID	NO: 197:

CTG Leu -5	GTT Val	CCG Pro	CGT Arg	GGA Gly	TCC Ser 1	CCG Pro	GCT Ala	CCG Pro	CCA Pro 5	GCT Ala	TGT Cys	GAC Asp	CTT Leu	CGT Arg 10	GTT Val	48
CTG Leu	TCT Ser	AAA Lys	CTG Leu 15	CTT Leu	CGC Arg	GAC Asp	TCT Ser	CAC His 20	GTG Val	CTG Leu	CAC Hie	TCT Ser	CGT Arg 25	CTG Leu	TCC Ser	96
CAG Gln	TGC Cys	CCG Pro 30	GAA Glu	GTT Val	CAC His	CCG Pro	CTG Leu 35	CCG Pro	ACC Thr	CCG Pro	GTT Val	CTG Leu ·40	CTT Leu	CCG Pro	GCT Ala	144
										CAG Gln						192
						Ala				CTT Leu 70						240
										CTG Leu						288
										GGC Gly						336
										ACC Thr						384
										CTG Leu					GTG Val	432
										CTC Leu 150						480
										TCT Ser						528
	GAG Glu			TGAG.	TAA	TC		•								549

### What is claimed is:

- 1. A thrombopoietin (TPO) polypeptide having the biological activity of specifically stimulating or increasing platelet production and comprising the amino acid sequence 1 to 332 of SEQ ID NO:6 or derivative thereof.
- 2. The TPO polypeptide derivative according to claim 1 consisting of the amino acid sequence 1 to 163 of SEQ ID NO:6.
- 3. The TPO polypeptide derivative according to claim 1 consisting of the amino acid sequence 1 to 232 of SEQ ID NO:6.
- 4. The TPO polypeptide derivative according to claim 1 consisting of the amino acid sequence 1 to 151 of SEQ ID NO:6.
- 5. The TPO polypeptide derivative of claim 1, 2 or 3 having from 1 to 6 amino terminal amino acids deleted.
- 6. The TPO polypeptide derivative according to claim 2 selected from the group consisting of  $[\Delta His^{33}]$ TPO (1-163),  $[\Delta Arg^{117}]$ TPO (1-163) and  $[\Delta Gly^{116}]$ TPO (1-163).
- 7. The TPO polypeptide derivative according to claim 2 selected from the group consisting of [His $^{33}$ , Thr $^{33}$ ', Pro $^{34}$ ]TPO (1-163), [His $^{33}$ , Ala $^{33}$ ', Pro $^{34}$ ]TPO (1-163), [Gly $^{13}$ , Asn $^{116}$ ', Arg $^{117}$ ]TPO (1-163), [Gly $^{116}$ , Ala $^{116}$ ', Arg $^{117}$ ]TPO (1-163), [Gly $^{116}$ , Gly $^{116}$ ', Arg $^{117}$ ]TPO (1-163) and [Ala $^{1}$ , Val $^{3}$ ]TPO (1-163).

- 8. The TPO polypeptide derivative according to claim 1 selected from the group consisting of [Thr<sup>33</sup>, Thr<sup>333</sup>, Ser<sup>334</sup>, Ile<sup>335</sup>, Gly<sup>336</sup>, Tyr<sup>337</sup>, Pro<sup>338</sup>, Tyr<sup>339</sup>, Asp<sup>340</sup>, Val<sup>341</sup>, Pro<sup>342</sup>, Asp<sup>343</sup>, Tyr<sup>344</sup>, Ala<sup>345</sup>, Gly<sup>346</sup>, Val<sup>347</sup>, His<sup>348</sup>, His<sup>349</sup>, His<sup>350</sup>, His<sup>351</sup>, His<sup>352</sup>, His<sup>353</sup>]TPO and [Asn<sup>25</sup>, Lys<sup>231</sup>, Thr<sup>333</sup>, Ser<sup>334</sup>, Ile<sup>335</sup>, Gly<sup>336</sup>, Tyr<sup>337</sup>, Pro<sup>338</sup>, Tyr<sup>339</sup>, Asp<sup>340</sup>, Val<sup>341</sup>, Pro<sup>342</sup>, Asp<sup>343</sup>, Tyr<sup>344</sup>, Ala<sup>345</sup>, Gly<sup>346</sup>, Val<sup>347</sup>, His<sup>348</sup>, His<sup>349</sup>, His<sup>350</sup>, His<sup>351</sup>, His<sup>352</sup>, His<sup>353</sup>]TPO.
- 9. The TPO polypeptide derivative according to claim 1 selected from the group consisting of [ $Asn^{25}$ ]TPO and [ $Thr^{33}$ ]TPO.
- 10. The TPO polypeptide derivative according to claim 2 selected from the group consisting of [Ala¹, Val³, Arg¹29]TPO (1-163), [Ala¹, Val³, Arg¹33]TPO (1-163), [Ala¹, Val³, Arg¹43]TPO (1-163), [Ala¹, Val³, Leu<sup>82</sup>]TPO (1-163), [Ala¹, Val³, Leu¹46]TPO (1-163), [Ala¹, Val³, Pro¹48]TPO (1-163), [Ala¹, Val³, Arg¹5]TPO (1-163) and [Ala¹, Val³, Arg¹15]TPO (1-163).
- 11. The TPO polypeptide derivative according to claim 2 selected from the group consisting of [Arg129]TPO (1-

- 163), [Arg<sup>133</sup>]TPO (1-163), [Arg<sup>143</sup>]TPO (1-163), [Leu<sup>82</sup>]TPO (1-163), [Leu<sup>146</sup>]TPO (1-163), [Pro<sup>148</sup>]TPO (1-163), [Arg<sup>59</sup>]TPO (1-163) and [Arg<sup>115</sup>]TPO (1-163).
- 12. The TPO polypeptide according to claim 1 covalently bonded to a polymer.
- 13. The TPO polypeptide according to claim 12 wherein said polymer is polyethylene glycol.
- 14. The TPO polypeptide according to any of claims 1 through 13 which further comprises the amino acid Met<sup>-2</sup>-Lys<sup>-1</sup>.
- 15. The TPO polypeptide according to any of claims 1 through 13 which further comprises Met<sup>-1</sup>.
- 16. The TPO polypeptide according to any claims 1 through 13 which further comprises Gly<sup>-1</sup>.
- 17. A polypeptide consisting of the mature sequence of amino acids of SEQ ID NOs:2, 4 or 6.
- 18. A DNA encoding a TPO polypeptide according to any of claims 1 through 11, 14, 15 and 17.

- 19. A DNA sequence for use in securing expression of a TPO polypeptide having the biological activity of specifically stimulating or increasing platelet production, which is selected from the group consisting of:
- (a) the DNA sequences shown in SEQ ID NOS:194, 195 or 196 or complementary strands thereof; and
- (b) DNA sequences which hybridize under stringent conditions to the DNA sequences as defined in (a) or fragments thereof; and
- (c) DNA sequences which would hybridize to the DNA sequences as defined in (a) and (b), but for the degeneracy of the genetic code.
  - 20. A cDNA sequence according to claim 19.
- 21. A genomic DNA sequence according to claim 19.
- 22. A manufactured DNA sequence according to claim 19.
- 23. A process for producing a TPO polypeptide comprising the steps of:

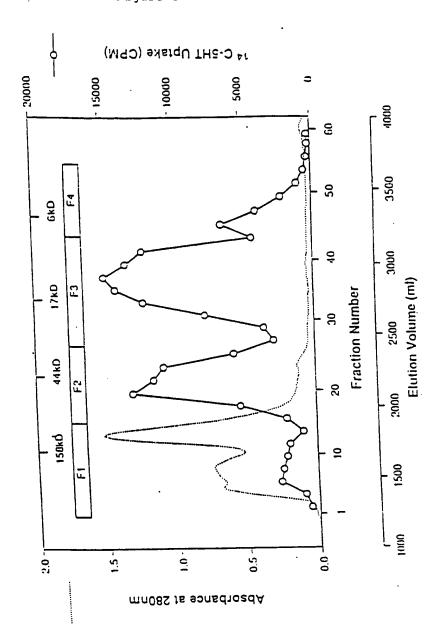
- a) expressing a polypeptide encoded by a
   DNA according to any of claims 18, 19,
   20, 21 or 22 in a suitable host, and
- b) isolating said TPO polypeptide.
- 24. The process of claim 23 wherein the TPO polypeptide expressed is a Met<sup>-2</sup>-Lys<sup>-1</sup> polypeptide and further including the step of cleaving Met<sup>-2</sup>-Lys<sup>-1</sup> from said isolated TPO polypeptide.
- 25. A DNA sequence for use in securing expression of a TPO polypeptide having the biological activity of specifically stimulating or increasing platelet production, said DNA further encoding a thrombin recognition peptide 5' to TPO polypeptide encoding sequences and encoding glutathione-S-transferase (GST) 5' to the thrombin recognition peptide.
- 26. The DNA sequence of claim 25 for use in securing expression of a TPO polypeptide consisting of the amino acid sequence 1 to 174 of SEQ ID NO:6.
- 27. A process for producing a TPO polypeptide having the biological activity of specifically stimulating or increasing platelet production comprising the steps of:

- a) expressing a polypeptide encoded by a
   DNA according to claim 25 or claim 26
   in a suitable host, and
- b) isolating said GST-(thrombin recognition peptide)-TPO polypeptide.
- c) treating said isolated GST-(thrombin recognition peptide)-TPO polypeptide with thrombin, and
  - d) isolating a Gly-1-TPO polypeptide.
- 28. The process of claim 27 wherein the Gly<sup>-1</sup>-TPO polypeptide is [Gly<sup>-1</sup>]TPO (1-174).
- 29. The process of claim 27 or claim 28 wherein the isolated Gly-1-TPO polypeptide is a TPO derivative consisting of the amino acid sequence 1 to 174 of SEQ ID NO:6.
- 30. A protein product obtained by the process of claims 23, 24, 27, 28 or 29.
- 31. A procaryotic or eucaryotic host cell transformed or transfected with the DNA sequence according to any of claims 18, 19, 20, 21, 22, 25 or 26 in a manner enabling said host cell to express a polypeptide having the biological activity of specifically stimulating or increasing platelet production.

- 32. A pharmaceutical composition comprising an effective amount of the polypeptide according to any of claims 1 through 16 and 30 in combination with a pharmaceutically acceptable carrier.
- 33. A pharmaceutical composition according to claim 32 for use in the treatment of platelet disorders.
- 34. A pharmaceutical composition according to claim 32 for use in the treatment of thrombocytopenia.
- 35. A pharmaceutical composition according to claim 34 for use in the treatment of thrombocytopenia induced by chemotherapy, radiotherapy or bone marrow transplantation.
- 36. A method for treating platelet disorders, which comprises administering an effective amount of the polypeptide according to any of claims 1 through 16 and 30 to patients having the disorders.
- 37. A method for treating thrombocytopenia, which comprises administering an effective amount of the polypeptide according to any of claims 1 through 16 and 30 to patients having thrombocytopenia.

- 38. A method according to claim 37 for treating thrombocytopenia induced by chemotherapy, radiotherapy or bone marrow transplantation.
- 39. An antibody specifically immunoreactive with a polypeptide of any claims 1 through 16 and 30.
- 40. Use of the antibody of claim 39 for the isolation of a polypeptide according to any of claims 1 through 16 and 30.
- 41. Use of the antibody of claim 39 for the quantification of a polypeptide according to any of claims 1 through 16 and 30.

Figure 1



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Figure 2

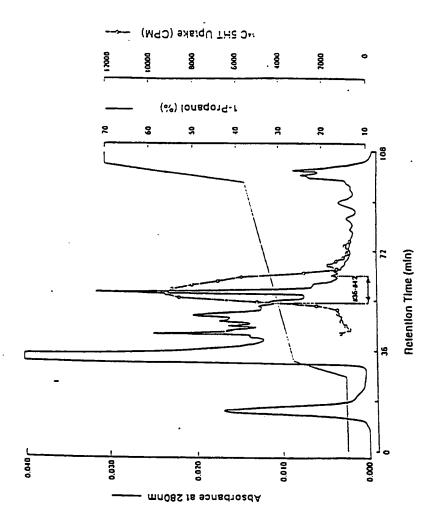


Figure 3 -

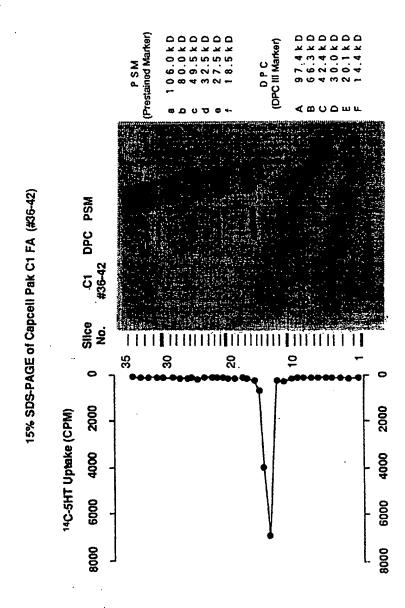
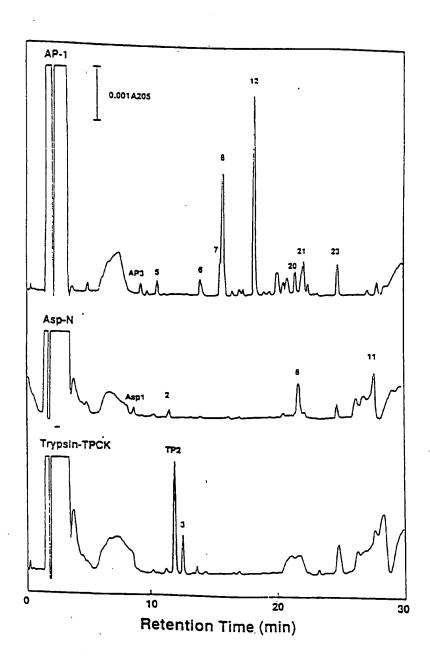
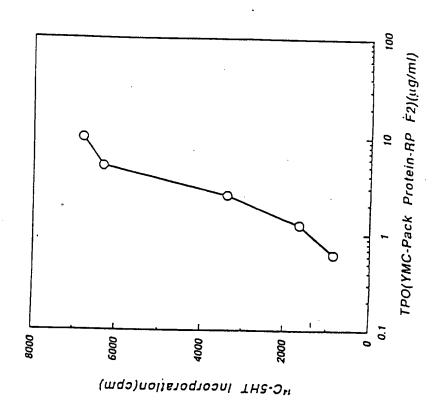


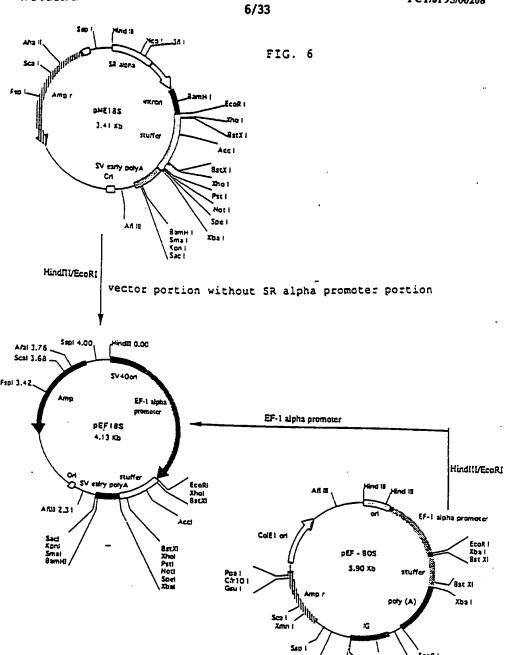
FIG. 4



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Figure 5





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Figure 7

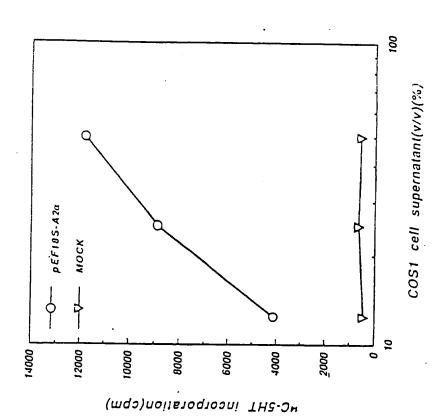


Figure 8

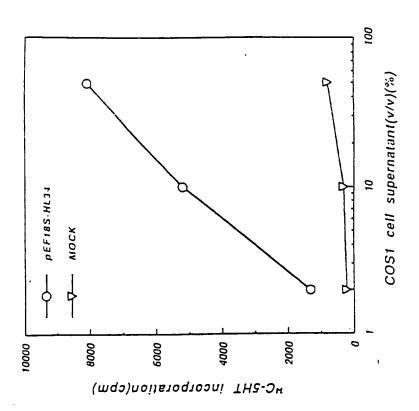


Figure 9

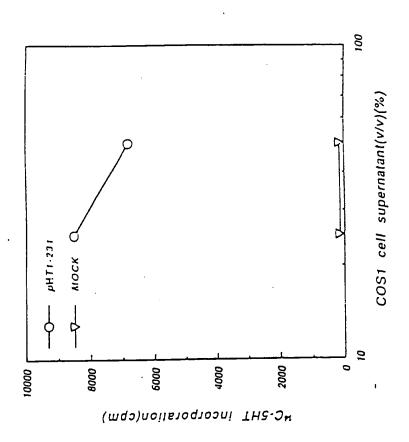
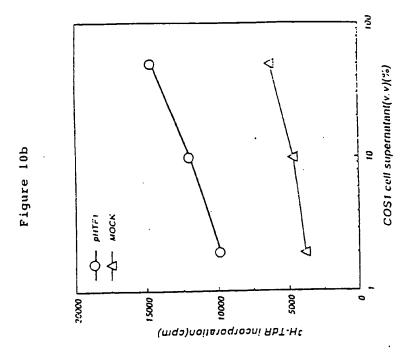
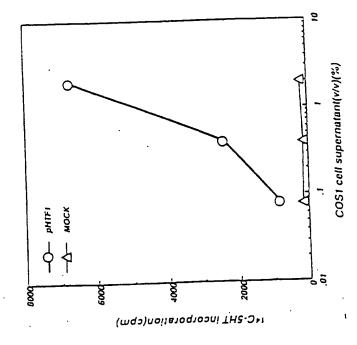


Figure 10a





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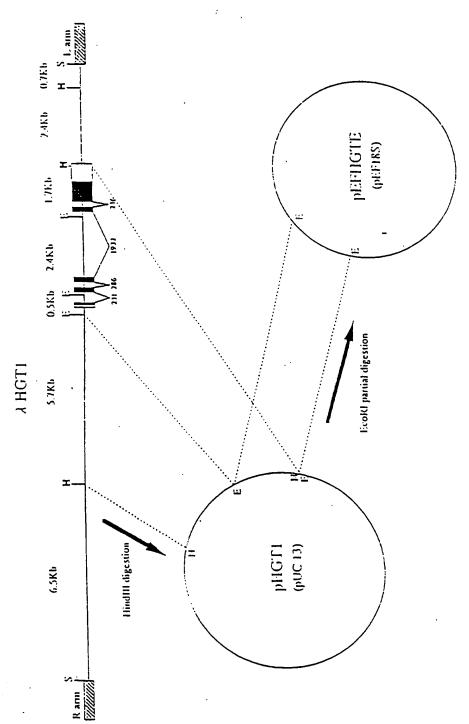


Figure 11

Figure 12a

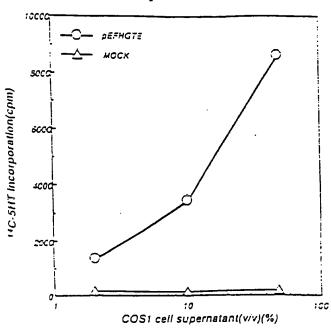
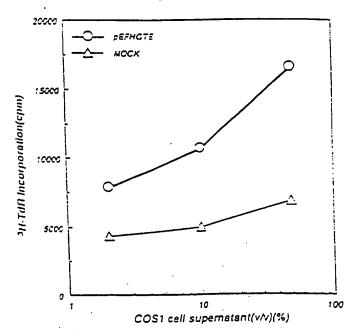


Figure 12b



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Figure 13a

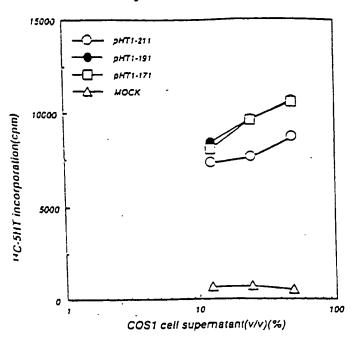


Figure 13b

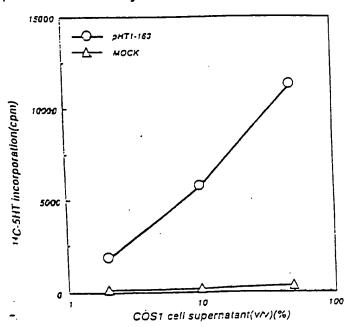
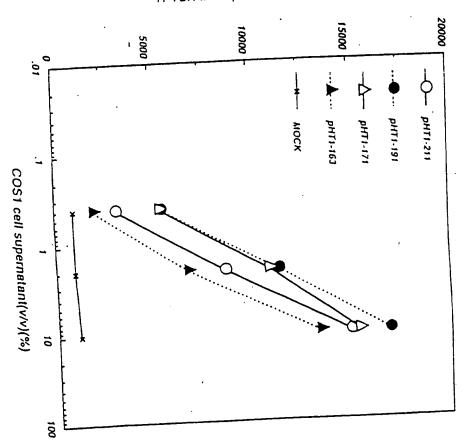
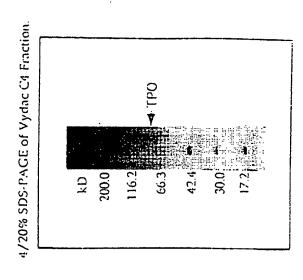


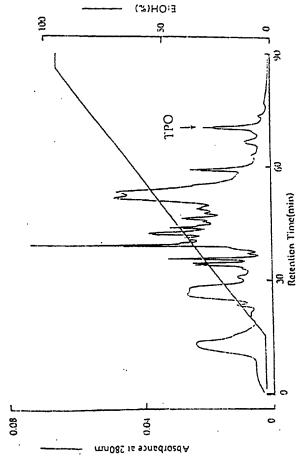
Figure 14

<sup>3</sup>H-TdR incorporation(cpm)









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Figure 17

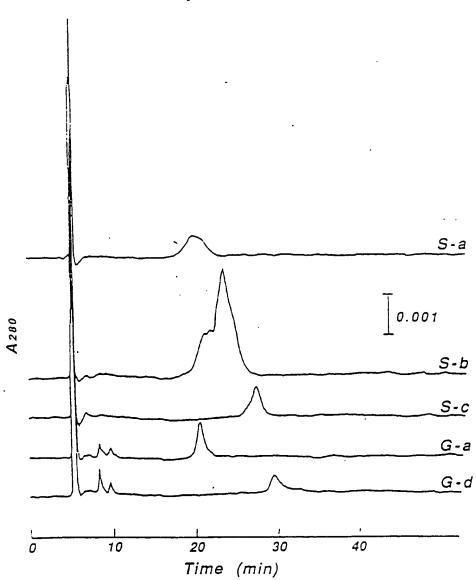
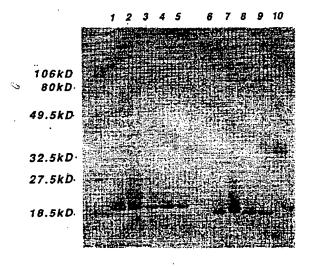


Figure 18



1,6 : S-a

2,7 : S-b 3,8 : S-c

4,9 : G-8

5,10 : G-d

1-5, DTT+, 6-10 DTT-

Figure 19

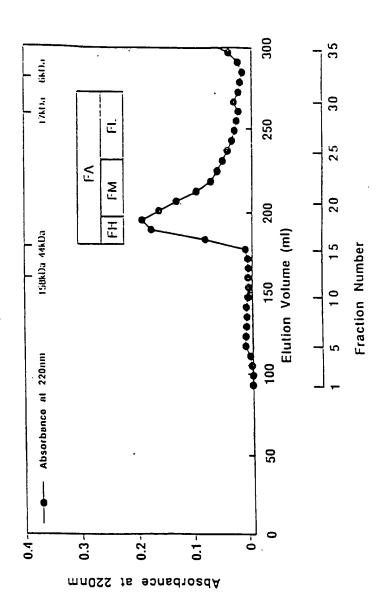


Figure 20

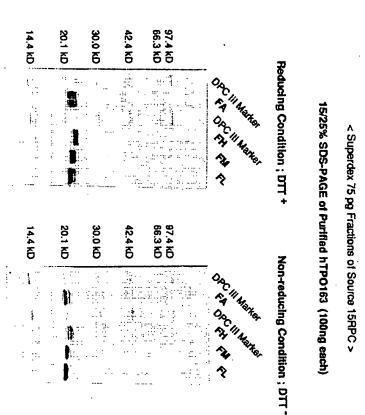


Figure 21

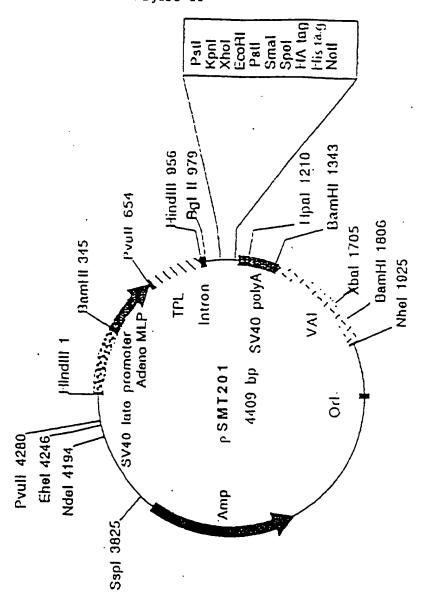
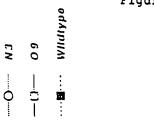
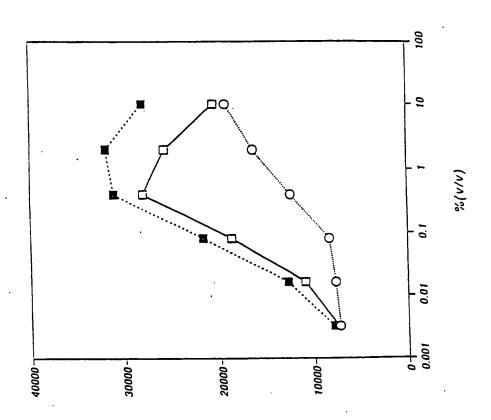


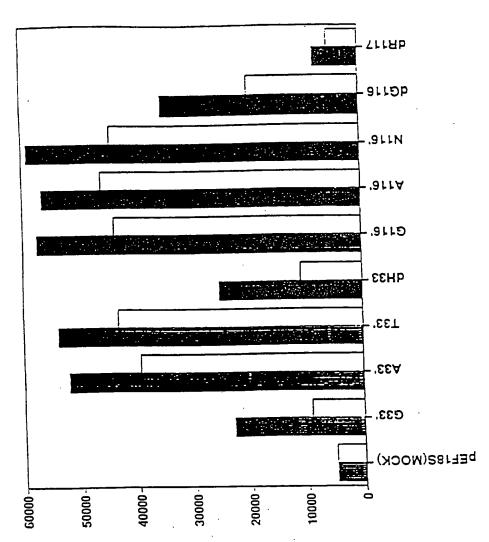
Figure 22



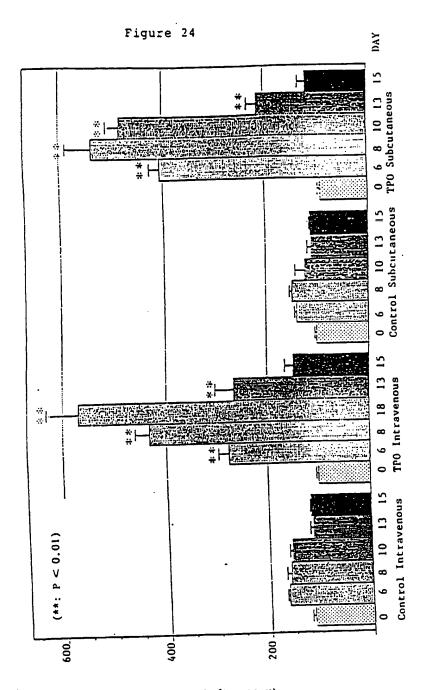


(cpm) noistiononi RbT-HE

Figure 23



aH-TdR Incorporation (cpm)



Placeler Counts (x 10000/µl)

m. Lightness .

Figure 25

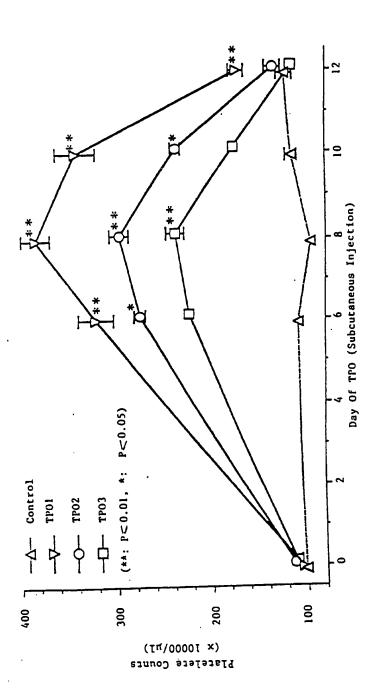


Figure 26

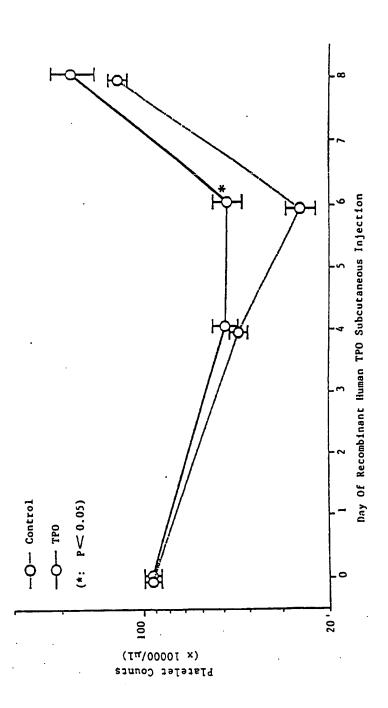


Figure 27

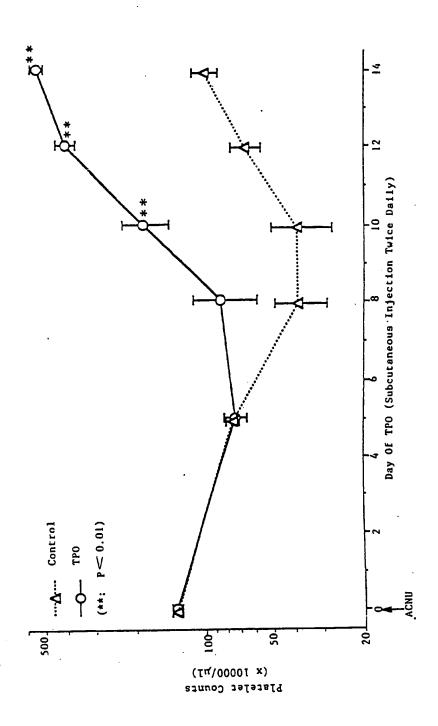


Figure 28

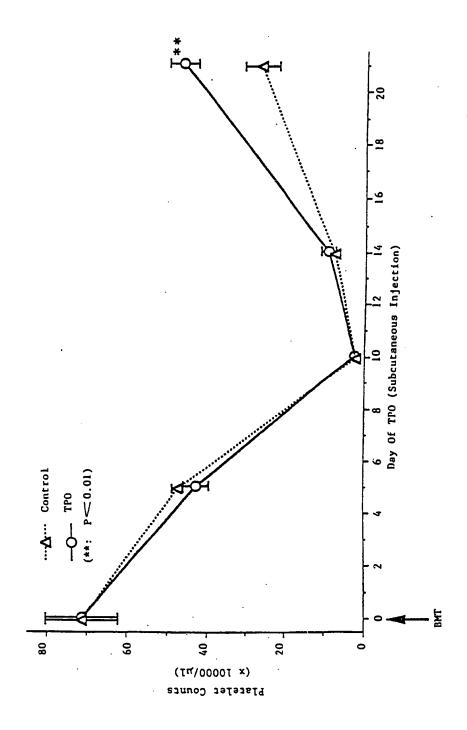


Figure 29

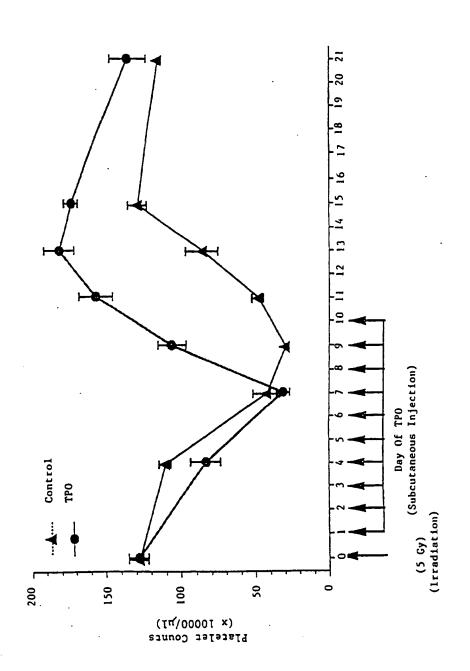


Figure 30

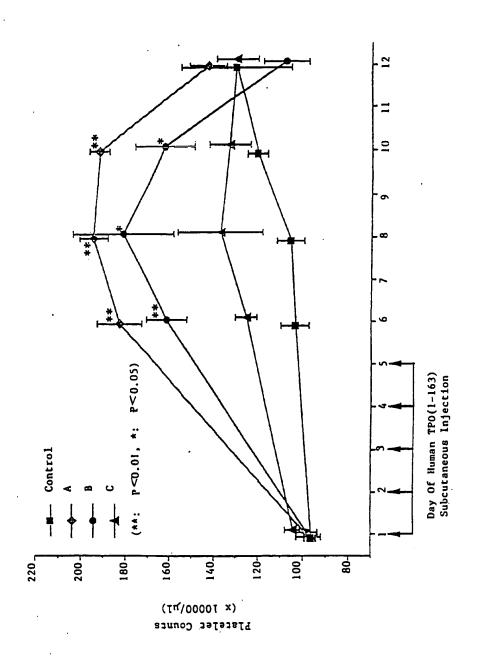


Figure 31

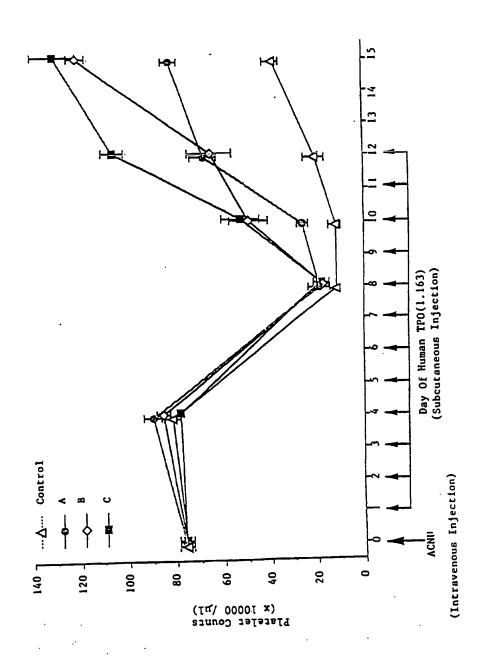


Figure 32

MPL-X completely blocks the ability of APK9 to induce megakaryocyte development

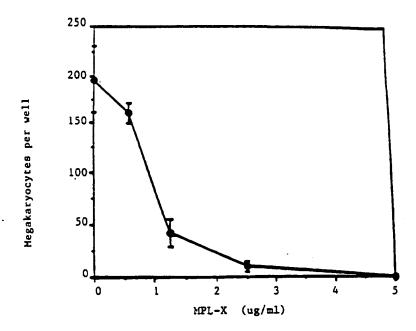


Figure 33

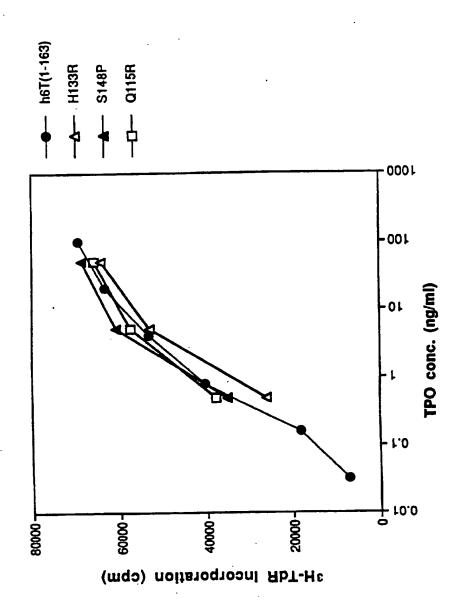


Figure 34

